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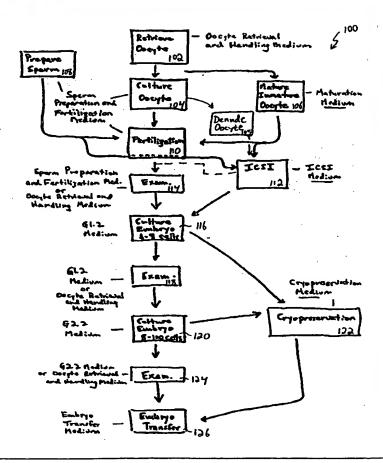
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(54) Title: SYSTEM AND SEQUENTIAL CULTURE MEDIA FOR IN VITRO FERTILIZATION

(57) Abstract

Instead of immersing human reproductive cells in a single culture medium throughout the various procedures used in IVF, a process is provided by which the reproductive cells may be moved through a sequence of distinct culture media as the various IVF procedures are carried out. In one implementation, the culture media specifically formulated to provide a physical environment similar to that found within the female reproductive tract and conducive to growth and development of human reproductive cells during the various stages of the IVF process. In this regard, specifically formulated culture media can be applied to support the reproductive cells in one or more of the following procedures: oocyte retrieval and handling; oocyte maturation; ordinary fertilization; oocyte, zygote and embryo examination and biopsy; embryonic development to the eight-cell stage; embryonic development to the blastocyst stage; embryo transfer, and cryopreservation.



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SYSTEM AND SEQUENTIAL CULTURE MEDIA FOR IN VITRO FERTILIZATION

FIELD OF THE INVENTION

The present invention relates generally to human in vitro fertilization (IVF) and, in particular, to a sequential culture media system and process to be used in oocyte retrieval, handling and maturation, sperm preparation, fertilization, embryo development and transfer, and cryopreservation. The invention provides the gametes, zygote and developing embryo with a physical environment adapted to their physiological needs, so supporting their normal growth and development in vitro and increasing the likelihood of successful pregnancy.

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BACKGROUND OF THE INVENTION

In vitro fertilization seeks to duplicate, to a large extent, the conditions and processes normally occurring within the female reproductive system that are necessary to oocyte development, fertilization and early embryonic development. In the clinic and laboratory, IVF involves several discrete procedures, such as collection of the oocytes from the ovary of the mother, preparation of the sperm, fertilization, and, once fertilized eggs are identified, a period of early embryonic development, and then transfer of the embryo to the mother's uterus. Each of these steps can take place over extended periods of time, during which the individual cells involved have a continuing need for nutrients, and are subjected to significant stress as a result of clinical manipulation and changed environmental conditions.

During IVF, a culture medium is ordinarily used as a substitute for the fluid sime. secreted by the female reproductive tract that would ordinarily surround the gametes, in his our of zygote, and developing embryo. Most laboratories carrying out IVF use a single culture medium throughout the various procedures involved: In a number of GENT FIRST laboratories, there has been a tendency to use tissue culture media for IVF procedures, such as Ham's F-10, which is formulated to support somatic cell growth, not gamete or embryonic cell growth. Tissue culture media generally are complicated systems, containing an array of amino acids, vitamins and other constituents. They can contain components that significantly impair embryonic development and viability after ate anoth diasts ye work transfer. Further, to the extent tissue culture media contain components that are 600,6003

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generally needed by the gametes and the embryo, the media are not formulated to provide the components at levels appropriate to support healthy gamete and embryonic development.

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Other laboratories have used simple culture media, consisting of balanced salt solutions supplemented with carbohydrate energy sources such as glucose, pyruvate and lactate. Examples include Earle's, T-6, and human tubal fluid (HTF). These media generally lack certain key components found in the female reproductive tract, such as non-essential amino acids, and their constituents are not formulated in concentrations that meet the specific changing needs of the gametes and developing embryo at various stages of their development.

The two types of culture media commonly used for IVF generally are only capable of supporting embryonic development to the eight-cell stage. Such media are ordinarily not capable of supporting and producing a viable blastocyst stage embryo, complete with an epithelium and competent inner cell mass. Accordingly, embryo transfer, the return of the fertilized oocyte to the uterus of the mother, usually occurs at around the four-cell stage (day two) or around the eight-cell stage (day three). This is a time when the four- or eight-cell embryo would not typically have arrived in the uterus of the mother, if fertilization had occurred in vivo. Embryo transfer at this time involves placing the cleavage stage embryo in an environment oriented to a blastocyst stage embryo. The cleavage stage embryo must then undergo further development in a non-homologous environment to reach the blastocyst stage, in which the embryo has trophectoderin cells capable of implanting in the uterine lining.

Recent research and human trials have led to the development of two new culture media, G1 and G2, which represent significant advancements in adaptation of culture media to the physiological needs of the cleavage stage embryo and the embryo in the eight-cell through blastocyst stage of development. These media are described in the following publications: Barnes, Crombie, Gardner, et al, Blastocyst Development and Birth After In-vitro Maturation of Human Primary Oocytes, Intracytoplasmic Sperm Injection and Assisted Hatching, Human Reproduction, vol. 10, no. 12, pp. 3243-47 (December, 1995); Gardner and Lane, Culture and Selection of Viable Blastocysts: A Feasible Proposition for Human IVF?, Human Reproduction Update, Vol. 3, No. 4, pp. 367-82 (1997); Gardner, Vella, Lane, et al, Culture and

Transfer of Human Blastocysts Increases Implantation Rates and Reduces the Need for Multiple Embryo Transfers, Fertility and Sterility, Vol. 69, No. 1, pp. 84-88 (January 1998). Use of these media, and particularly the G2 medium, supports the growth and development of viable blastocyst stage embryos in vitro. Accordingly, the development of these media paves the way for new approaches to embryo transfer to the uterus at the blastocyst stage, when the embryo is most adapted to surviving in the uterine environment and has developed structures and capabilities required for implantation to take place. Research utilizing the G1 and G2 media, and embryo transfer at the blastocyst stage, suggests that these media contribute to higher pregnancy rates, and reduces the need for transfer of multiple embryos and the risk of multiple births. Neither of these media, however, is optimized for supporting the gametes, oocyte maturation, or fertilization.

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SUMMARY OF THE INVENTION TO THE PROPERTY OF TH

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It has been recognized that IVF processes may be improved by providing specific media and media sequences for supporting gametes, zygotes and developing embryos relative to various phases of the IVF process. In certain respects, such media and sequences better reflect in vivo development. Within the female reproductive and sequences better reflect in vivo development. system, the oocyte is developed within and released from the ovary during ovulation, and proceeds through the oviduct towards the uterus. During this journey, it and the state of the experiences a dynamic physical environment. The fluid of the oxiduct contains a crowd and number of components that provide nourishment to the oocyte and its surrounding control secul miral destination cumulus cells, and that also appear to interact with the oocyte and its cumulus cells, so stimulating development. Similarly, the fluid of the female reproductive tracted and the provides nourishment to sperm traveling through the oviduct, and also stimulates even and also stimulates THE ROBBINS OF SERVICES certain changes in the sperm necessary to fertilization. Once fertilization occurs, the hand ระเถลมที่ ด้วย 64年1月8日本 11年1月1日 resulting zygote travels down the oviduct and enters the uterus approximately three of the L mouth our parise contin days later, undergoing internal transformation and experiencing a changing transformation and experience and exper environment. the ending of the control of the problem of the second of the control of the cont

As the zygote travels, cell division, or cleavage, occurs as well as significant developmental changes. The cells of early embryonic development have different capabilities and nutritional needs from those of later embryonic development prior to a significant development development prior to a significant development development prior to a significant development develop

implantation. The zygote and cleavage stage embryo (up to the eight-cell stage) are characterized by low levels of biosynthesis, low respiratory rates, only limited ability to metabolize glucose, and a capacity to utilize pyruvate. As the embryo develops, and genome activation occurs, the embryo gains an increased capacity to utilize glucose. At the blastocyst stage of development, when the embryo is entering and within the uterus, the embryo's metabolic system has developed and the embryo has a substantially greater capacity to use and need for glucose, and less need for pyruvate. The makeup of the fluid surrounding the developing embryo in the female reproductive tract seems to be tailored to these changing needs: in the oviduct at the time when the oocyte and developing embryo are present, relatively low levels of glucose are found, while pyruvate concentrations are high; at the time the embryo enters the uterus, glucose reaches its highest level and the pyruvate concentration is comparatively low. Cleavage stage embryos, like the oocyte, are susceptible to loss of amino acids through their cell membranes when surrounded by an environment having a low concentration of such factors. Such loss of internal amino acids can have devastating effects. Again, as if in response to these needs of the osmolyte sensitive oocyte and cleavage stage embryo, the female reproductive tract typically has high levels of specific amino acids that are very similar to those found in the oocyte and cleavage stage embryo

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In view of the foregoing, an important object of the present invention is to further improve and enhance the culture of human reproductive cells in vitro. The invention is intended to promote the health and viability of the gametes, zygote and embryo at various stages of the IVF process, thereby improving the overall efficiency of the IVF process and increasing pregnancy rates.

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In general, the present invention involves the application of separate media specifically formulated to meet the physiological needs of the gametes, zygote and/or developing embryo in various stages of their development, and to support the processes necessary to accomplish fertilization and embryonic development in vitro. The present invention also generally contemplates a sequential culture media system, in which the separate media utilized have integrated formulations, intended to minimize trauma to the reproductive cells as they are moved from one medium to another during the IVF process.

In one aspect of the present invention, an oocyte retrieval and handling medium is provided for use in the clinical procedure of retrieving the oocyte from the mother. The medium may be used for flushing, washing and holding the oocyte was during the process of removing the occyte from the mother's overy, and for storing the oocyte for a period prior to fertilization. An optional use of the medium envisioned by the invention is with procedures where handling or manipulating the oocyte, with zygote, or embryo is necessary, such as examination of the oocyte to determine whether fertilization has occurred, or examining the embryo to determine the progression of its development. The present invention includes use of an oocyte retrieval and handling medium comprised of water, ionic constituents, and a buffer. Preferably the buffer used in the medium is 4-Morpholinepropanesulfonic acide with (MOPS) or N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid (HEPES).: Instruction addition, the medium may be supplemented with the carbohydrates glucose, lactate and pyruvate. The medium may be supplemented with non-essential amino acids. An optional formulation of the medium, lacking calcium and magnesium, may be used in biospsy procedures. Another optional formulation of the medium includes antibiotics, such as penicillin and/or streptomycin, to destroy bacteria that might be introduced into the medium during the process of oocyte collection. A contract to all value and

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Another aspect of the present invention involves the provision and use of another occyte maturation medium, for example, in circumstances where the pocyte is removed from the mother before it is mature. An example of a situation where application of this medium may be desired arises when it is necessary to treat the cocytes collected from the mother with hormones in vitro due to the mother's intolerance of such hormones. The invention contemplates holding the promote development prior to fertilization. An optional use of the maturation medium implicated accordance with the invention is for collection, although the most cost effective approach will normally involve use of the retrieval and handling medium for initial approach will normally involve use of the retrieval and handling medium for initial approach will normally involve use of the retrieval and handling medium for initial approach will normally involve use of the retrieval and handling medium for an extended period prior to fertilization. The present invention contemplates use of a maturation medium comprised of water, ionic constituents, and a buffer.

Preferably, the maturation medium is supplemented with the carbohydrates glucose,

lactate and pyruvate. Specific formulations in accordance with the present invention may involve successive supplementation of the medium with one or more of the following: non-essential amino acids; essential amino acids; cysteamine; human serum albumin (HSA) and hyaluronate; one or more growth factors such as insulin transferin selenium (ITS), insulin-like growth factor (IGF), and epidermal growth factor (EGF); and hormones follicule stimulating hormone (FSH) and human chorionic gonadotrophin (hCG).

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Another aspect of the invention involves the provision and use of a sperm preparation and fertilization medium. This medium may be applied to wash, prepare, and store sperm, to store the oocyte in the period prior to fertilization, and to serve as the medium in which the sperm and oocyte are placed together and fertilization occurs. The present invention contemplates use of a sperm preparation and fertilization medium that includes water, ionic constituents, and a buffer. Preferably, the medium contains an elevated concentration of sodium, as compared to the oocyte retrieval and handling medium, to promote sperm function and fertilization. In addition, the medium may be supplemented with an elevated phosphate concentration, as compared to the oocyte retrieval and handling medium. Even more preferably the medium is supplemented with the carbohydrates glucose, lactate and pyruvate. Specific formulations may involve supplementation of the medium with one or more of: bicarbonate; glutathione to promote sperm head decondensation; non-essential amino acids; HSA and hyaluronate; and antibiotics such as penicillin and streptomycia.

sperm injection (ICSI) and related methodology. The ICSI procedure may be necessary where there are obstacles to normal fertilization, such as a thickened zona pellucida on the oocyte hindering sperm head penetration. ICSI involves removal of the cumulus cella and injection of the sperm into the oocyte, ordinarily through a glass pipette. The invention contemplates placing sperm in the ICSI medium, capturing the sperm by drawing the medium containing sperm into the pipette, inserting the pipette containing medium and sperm into the oocyte, and, following insertion into the oocyte, transferring the medium containing sperm from the pipette into the oocyte. The ICSI medium used in the present invention includes the constituents water, ionic

constituents and a buffer. Preferably, in the present invention the medium lacks phosphate. More preferably, the buffer used in the medium is MOPS or HEPES. Additionally, the medium may be supplemented with the carbohydrates lactate and pyruvate and the medium may be further supplemented with one or more of the non-essential acids most abundant in the oocyte: glutamine, glycine, proline, serine, and taurine. In one formulation, the ICSI medium used is supplemented with hyaluronate or polyvinylpyrolidone (PVP) to slow or immobilize the sperm so that they may be captured by pipette for the ICSI process. Further, an alternative formulation of the ICSI medium referred to as denuding medium used in the invention includes hyaluronidase, which is included in the portion of the medium used to denude the oocyte prior to the ICSI process.

Another aspect of the present invention involves the provision and use of a medium for supporting initial cell cleavage and embryonic development following fertilization, the medium herein referred to as G1.2. The invention contemplates washing the inseminated oocyte and zygote in the medium and placing the zygote in the medium for a period of about 48 hours to support cell cleavage and development through about the eight-cell stage. The present invention involves use of a medium that includes the constituents water, ionic constituents, and a buffer. Preferably, the medium is supplemented with the carbohydrates glucose, lactate, and pyruvate. The medium may also be supplemented with non-essential acids. Specific formulations in accordance with the invention may include one or more of the following supplements: EDTA; HSA; and hyaluronate. The form of glutamine used in the medium is a clear preferably alanyl-glutamine, which is particularly stable and less properto breakdown to the waste product ammonium, which is toxic to the developing embryonics.

A further aspect of the invention involves the provision and use of a second medium for embryo development, herein referred to as G2.2. The invention contemplates placing the embryo in the G2.2 medium for a period of about 48 hours, or preferably at or after the eight-cell stage, and continuing through the blastocyst stage of development and up to the point of embryo transfer. This medium is specifically adapted for and has as its preferred use support of the embryo from the eight-cell stage through the time at which implantation occurs, in tandem with the use of G1.2. for initial embryonic development. The invention involves a G2:2 medium that

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includes water, ionic constituents, and a buffer. Preferably the medium is supplemented with the carbohydrates glucose, lactate and pyruvate. More preferably, as compared to medium G1.2, medium G2.2 is supplemented with depressed levels of lactate and pyruvate, and elevated levels of glucose. Additionally, the medium may be supplemented with the non-essential amino acids, except taurine. Specific formulations in accordance with the present invention involve supplementing the medium with one or more of: essential amino acids, which stimulate development of the inner cell mass of the blastocyst; vitamins, which further facilitate the function of the blastocyst; HSA; and hyaluronate. An important aspect of the G2.2 medium, in all formulations, is the absence of EDTA.

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Another aspect of the invention is the provision and use of an embryo transfer medium. The invention contemplates that this medium will be used as a carrier for the embryo when it is transferred back into the mother. The invention may involve the same formulations of the medium for embryo transfer as are used with medium G2.2. More preferably for embryo transfer, however, the formulation of G2.2 is supplemented with a higher concentration of hyaluronate, which supports implantation of the embryo in the mother's uterus.

A further aspect of the invention is the provision and use of a medium for cryopreservation of the embryo and/or oocyte. The invention contemplates that the embryo may be placed in the medium at either the one- to eight-cell stage or eight-cell to blastocyst stage, and then frozen and stored in the medium. The invention also contemplates the che reedium may be used for cryopreservation of the oocyte. The cryopreservation medium contains ionic constituents, and a buffer. Preferably, it contains the MOPS or HEPES buffer. More preferably, it contains the carbohydrates lactate, pyruvate and glucose. Even more preferably, it contains HSA. Most preferably, the medium contains certain additives such as glycerol, ethylene glygol, DMSO and/or sucrose:

According to a further aspect of the invention, different media are used for two different phases of the IVF process, such as oocyte collection and maturation, sperm preparation, fertilization, embryo development and/or embryo transfer. One associated process involves obtaining a gamete from a first medium and introducing the gamete into a second medium different from the first medium, wherein

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fertilization occurs in the second medium. The step of obtaining a gamete from a first medium may include extracting an oocyte from an oocyte collection medium or oocyte maturation medium as described above. Additionally or alternatively, the step of obtaining may involve extracting sperm from a sperm preparation and fertilization medium as described above which, in turn, may be different from the oocyte medium. The step of introducing the gamete into the second medium may involve introducing the sperm and/or oocyte into a fertilization medium, or injecting sperm into an oocyte contained in the second medium. The various media may have integrated formulations for minimizing trauma to the reproductive cells.

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Another associated process in accordance with the present invention involves obtaining a zygote or embryo from a first medium wherein fertilization has occurred and introducing it into a second medium different from the first medium for a first growth phase. The first medium may be a fertilization medium as described above and the second medium may be the G1.2 medium as described above. The second medium may be used for supporting initial cell cleavage and embryonic development. The method may further involve transferring the resulting embryo from the second medium to a third medium for a second growth phase. The third medium may be a gentleman as described above.

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A further associated process in accordance with the present invention involves obtaining an embryo from a first medium and introducing the embryo into a second medium different from the first medium for transfer of the embryo into the mother for implantation. The first medium may be a G2.2 medium as described above and the second medium may be an embryo transfer medium as described above.

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For a more complete understanding of the present invention and further (terreform advantages thereof, reference is now made to the following detailed description taken and in conjunction with the drawings, in which: The land to the following detailed t

Figure 1 is a flowchart illustrating an IVF process in accordance with the accordance present invention.

DETAILED DESCRIPTION OF THE INVENTION OF

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The following description discloses the composition of various culture media in accordance with the present invention that are particularly adapted for use with IVF. Each of these media is specifically formulated to meet the physiological needs of the gametes, zygote and developing embryo at key points in the reproductive process. Also disclosed is a sequential culture media system. While each of the separate media could be used independently, the media also may be formulated together as a system, sharing a core group of ionic and non-essential amino acid constituents, with the objective of minimizing trauma to the oocyte, and the resulting zygote and embryo, as they are moved from one medium to another. The following description also discloses methods of using the media and the sequential culture media system in various clinical and laboratory procedures by which IVF is carried out, as well as methods of making the culture media.

A. Composition of the Sequential Culture Media

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1. Oocyte Retrieval and Handling Medium

A preferred oocyte retrieval and handling medium is an aqueous solution comprised of the ionic components sodium, potassium, phosphate, magnesium, bicarbonate, and calcium, to maintain an osmotic environment that does not stress the oocyte, and a buffering system, preferably MOPS or HEPES, to maintain the pH of the medium within the physiological range of 7.3 to 7.4. The ionic components are

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included in the preferred amounts depicted in column A of Table 1, and may be included in amounts described in the ranges depicted in column B of Table 1, 100%

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	NaLactate	(L-isomer)	10.5	5.0 - 20.0
15	NaPyruvate		0.32	0.1 - 1.0
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* Concentrations are in millimoles unless otherwise indicated; the medium is aqueous.

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It should be noted that Table 1 and the other tables presented in this section also describe the preferred form of the components used to make the respective culture media in practice. The MOPS buffer has not been used before in IVF procedures, and is preferred because it is not known to exhibit any toxic effects to reproductive cells and does not require maintenance of a CO₂ atmosphere above the medium. HEPES may also be utilized, although some research indicates a possible toxicity to reproductive cells. Table 1 depicts the preferred amount and ranges for the MOPS or HEPES buffer, although other buffering systems might be used. For example, a bicarbonate buffering system may be used because it is compatible with human

reproductive cells. Such a system would not ordinarily be practical with oocyte collection, because it requires the maintenance of elevated levels of CO₂ in the atmosphere surrounding the medium, which is ordinarily accomplished by use of a gassing incubator system that maintains a 3%-10% CO₂ atmosphere. Oocyte collection is a clinical procedure, in which it is typically not possible to maintain an elevated CO₂ atmosphere. In some clinical environments, such as where a humidicrib is available, it may be possible to perform oocyte collection in an elevated CO₂ atmosphere, and a bicarbonate buffer accordingly may be used. In accordance with the present invention, any buffering system used preferably maintains its buffering qualities during exposure of the medium to the atmosphere, and as well is preferably compatible with and not toxic to human reproductive cells.

The oocyte retrieval and handling medium also includes the carbohydrates glucose, lactate, and pyruvate, at levels similar to those found in the female reproductive tract at the corresponding point of ovulation. The preferred amounts and ranges in which these are found in the medium are depicted in Table 1. In addition, the preferred medium contains Eagle's non-essential amino acids (i.e., those not required for the development of somatic cells in culture) alanine, aspartate, asparagine, glutamate, glycine, proline, serine, and taurine, plus glutamine in the form of alanyl-glutamine, at levels similar to those found in the female reproductive system and in the oocyte. The preferred amounts and ranges are depicted in Table 1. The inclusion of non-essential amino acids and alanyl-glutamine in the medium is important to preventing osmotic shock; a medium lacking these components may drain the oocyte of its internal pool of amino acids, resulting in considerable intracellular trauma. An optional formulation of the medium which may be used in biopsy procedures, omits calcium and magnesium. Another optional formulation of the medium may include one or more antibiotics, such as penicillin and streptomycin, to destroy any bacteria that might be present around the oocyte or that might be introduced through the clinical procedure of oocyte removal.

2. Oocyte Maturation Medium

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The oocyte maturation medium is adapted for use with immature oocytes.

Oocyte maturation is typically used with mothers who are unable to withstand the

hormonal treatment ordinarily employed in IVF. Oocyte maturation generally involves treating the immature oocytes in vitro with the hormones follicle stimulating hormone (FSH) and human chorionic gonadotrophin (hCG) rather than injecting these hormones into the mother. The preferred medium is an aqueous solution that contains ionic constituents similar to those used in the oocyte retrieval and handling medium, at similar concentrations, although the magnesium level is increased and the calcium level decreased to maintain a 2:1 magnesium to calcium concentration. A buffer is included in the preferred medium to maintain a physiological pH. Because oocyte maturation ordinarily occurs in an incubator or isolette in which an elevated CO₂ atmosphere can be maintained, a bicarbonate buffering system is preferred. Other buffers may be used, provided they are compatible with the oocyteland other components of the medium. Table 2 provides the most preferred amounts of each of these components, as well as the preferred ranges of these components.

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to an hall through once of the consense Table 2 10 15 (12 cm) in the ex-

	Component Composition of Oocyte Maturation Medium*	
	me rievel element final cheminals de la Mist Preferred	<u>B</u> Preferred
	characteria prophibus quiene les commandes de Concentration	Rang
_	NaCl 90.08	80.0 - 100
5	J.J	3.5 - 7.5
	NaH,PO, 2H ₂ O Contributed and Applications of the Application of the	0.05 - 1.5
	£ .	0.2 - 4.0
10	CaCl ₂ .2H ₂ O	15 - 30.0 0.8 - 2.8
10	Glucose (L. 1971) englisated the field of the part of the 3.15 floor of the E. NaLactate (L. 190mer)	0.5 - 5.5
	NaLactate (L-isomer) NaPyruvate of baseline sea (%) and the last of the last	2.0 - 20.0
	V.1	0.01 - 1.0
16	Alanine Asparate O.1 The Hander And I was a construction of the C	0.01 - 0.5 0.01 - 0.5
15	Glutamate	0.01 - 0.5
	Alanyl - Glutamine of others in the state of	0.01 - 0.5
	Chroine	0.01 - 2.0 0.01 - 0.5
20	Proline the Late of the Control of t	0.01 - 0.5
20	Serine Cysteamine and the appropriate sends to expend the top to the bank of t	0.01 - 0.5
	Cysteamine 0.5 L-Arginine-HCl 0.6	0.1 - 2.0
	I-Cyctine 2HCl	0.1 - 1.2 0.05 - 0.25
25	L-Histidine-HCl-H2O 0.2	0.03 - 0.23
23	L-Isoleucine 0.4 L-Leucine 0.4 0.4 0.4 0.4 0.4	0.1 - 0.8
	I I min a IIO	0.1 - 0.8
	L-Methionine of and property as the property of the control of the	0.1 - 0.8 0.05 - 0.25
30	Trical and Jacobs of Cauchy and April 1989 1989 1989	0.1 - 0.4
50	V.4	0.1 - 0.8
	L-Tyrosine 2Na 0.2	0.1 - 0.9 0.1 - 0.4
	L-value by adodusted (5), to 3552672 (5), but he displayed in the first street of th	0.1 - 0.8
35	Choline Chloride is waited within the 21 feet of the children and the 1990 of	001 - 0.004
	Folic Acid 0.007	.003 - 0.01
	Folic Acid i-Inositof fam: during boxes, behave the royal of \$\begin{align*} 0.0023 \\ 0.00111 \end{align*}	
	Nacinamide A responsible that which is one of parents 0.0032 and the country which is 0.00	004 - 0.016
40	Riboflaving on the distance and the second of the second o	.002 - 0.01
	Thiamine HCl 0.003	001 - 0.0006
	Thiamine HCl 0.003. Perchant states of a cody hogo treates on a colored white state of the feet state of the colored states.	ήή1 ¹ .0.000
	HSA Hyaluronate on political the number quality of the 2 mg/ml. O 25mg/ml.	F - 10.0
•	o.zsngm	J.UD - U.D
4.5	ITS នៃសាសារបានប្រជាជា បានប្រជាជាមួយអ៊ី មាន បានបើប្រែស 10ng/ml	1 - 100
45	IGF-I Compositioned the adjustment of the Lating of the Lating Composition of the Composi	0 - 1000
	FGF 100ng/ml remains belong as the file of the Following state of the file of	0 - 1000
	PON	
	Temporal services and the services of the serv	
	* Concentrations are in millimoles, unless otherwise indicated; the medium is aqueous.	0.01∷- 10
	oncome and in minimoles, unless otherwise indicated; the medium is aqueous.	

The carbohydrates glucose, lactate and pyruvate are also included in the preferred maturation medium. Because of the presence and importance of cumulus cells that surround the developing oocyte, the glucose, lactate and pyruvate levels are adapted to the needs of the cumulus cells. Non-essential amino acids are preferably included in the medium to provide nutrients and avoid subjecting the oocyte to osmotic stress. Essential amino acids and vitamins may also be included to provide nutrients to the cumulus cells. The medium also contains HSA and hyaluronate. which act as a source of macromolecules. Insulin transferin selenium (ITS), insulinlike growth factor (IGF), and epidermal growth factor (EGF) are included to support the function of cumulus cells, which, in turn, nourish and stimulate the oocyte. FSH and hCG are added to stimulate the cumulus and oocyte to undergo changes dia del associated in vivo with ovulation. It should be noted that, when the maturation of the medium is prepared, ITS, IGF, EGF and FSH and hCG are preferably the last-added ingredients. The preferred amounts and ranges of these components are found in many components. Table 2.

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3. Sperm Preparation and Fertilization Medium

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Current methods of in vitro fertilization employ the same medium for sperm preparation and fertilization as is used for embryo development. No attempt has been made to develop a separate medium for preparation of sperm that is also suitable for storage and support of the oocyte, for promoting the process of fertilization, and for supporting the zygotes formed when fertilization occurs. In many laboratories, then fertilization process is allowed to take place over an extended period which ranges, from two to three hours to up to about sixteen (16) to eighteen (18) hours. During this time, the oocyte, sperm, and zygotes produced have significant nutritional needs. In addition, sperm function and fertilization tend to be encouraged when the surrounding fluid contains certain constituents. The sperm preparation and fertilization medium of the present invention is formulated to meet these concerns.

A preferred sperm preparation and fertilization medium in accordance with this invention has virtually the same composition of ions and non-essential amino acids as the oocyte retrieval and handling medium. The fact that these media share a similar ionic and amino acid composition minimizes the stress experienced by the

oocyte when it is removed from the retrieval and handling medium and placed in sperm preparation medium. Table 3 sets out the preferred amounts and ranges of the ionic and non-essential acid components.

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anona's	Mo	Compo	sition of S	Sperm	Prepar	ation a	nd F	erti	lizatio	n Med	liur	<u>n</u> *:	::1
	_		erett soos			<u> </u>				•			

Component A	num-dt :
ton	, <u>B</u> Preferred
concentration and the second of the second o	Range
NaClinary Ber i symbolic services and the services and the 100 common 2013.	a. Y. <u>25</u>
KCI	75-100
NaH ₂ PO4.2H2O distinctions of the many many different in a 5.5 minute on the specific of the control of the co	3.5 - 7.5
MgSO4:7H2O to the research on more by hours have accepted decimal and more	0.05 - 1.5
	0.2 - 4.0
NaLactate (L-isomer)	0.5 - 5.6
NaPyruvate selector to each time to select or in the first of 32 inches or the selection of	2.0 - 20
NaHCO3	0.1 - 0.5
AND THE COLOR OF BELLEVILLE MELLON PROPERTY OF THE COLOR	AT 15-30
$ ext{CaCl2.2H2O}$ ប្រគួមប្រហែងមែនអំពីនេះ ១៤៦ នេះ។ ដែលប្រសាសក $_{1.8}$ $^{1.5}$ $^{1.6}$ $^{1.6}$ $^{1.6}$ $^{1.6}$ $^{1.6}$	0.8 - 2.8
<u>analasta</u> ta galakwa sa motor tahun 1966 - a atanja ni ne	
Olutatilone 1.0mg/ml	0.5 - 5.0
្រាស់ ស្រាស់ ពេលក្នុង ដោយស្នាន់ ស្រាស់ ស្រាស់ ស្រាស់ ស្រាស់ ស្រាស់ មិន ស្រាស់ ស្រាស់ ស្រាស់ ស្រាស់ ស្រាស់ ស្រា	
Alanine Asparate	€0.01≎ 0.5
Asparagine sideli (2200) din e bi o e e e i o o e e e e e e e e e e e e	0.01 - 0.5
Glutamate settlement and the settlement of the s	0.01 - 0.5
0.1	0.01 - 0.5
Proline 0.1 Serine	0.01 - 0.5
Caurine 0.1 10 100 100 100 100 100 100 100 100	0.01 - 0.5
	,
asai redso ye mili, silibel deli prakos et ligil ni el trempe i capacit mili. ISA	
ISA Iyalumi of eldsur at nama econocive za film 5mg/ml 1 mg/ml 2 t 1 disc. Iyaluronate	atri 1.0 - 10.0
0.1mg/ml	0.02 - 0.5
enicillin spekara estano est gribme ora est. 0.06mg/ml	0.0110
treptomycin Teleptomycin Teleptomycin O.05mg/ml	6 1 0 0 1 10

* Concentrations are in millimoles unless otherwise indicated; the medium is aqueous.

As will be seen, the sperm preparation medium contains sodium at a higher concentration than the level found in the oocyte retrieval and handling medium. This elevated concentration of sodium promotes sperm function and fertilization, without causing undue osmotic stress to the oocyte. There is also a higher concentration of

phosphate, as compared to the occyte retrieval and handling medium. The glucose concentration of the sperm preparation and fertilization medium is elevated over that of the oocyte retrieval and handling medium, because glucose is the primary nutrient for sperm and cumulus cells around the egg. The lactate concentration of the present medium is lower than that found in the occyte retrieval and handling medium, to compensate for the tendency of sperm cells and cumulus cells to give off lactic acid as a waste product. A buffering system is used to maintain the physiological pH, and because sperm preparation and fertilization largely occur within an incubator that can maintain an elevated CO₂ atmosphere, a bicarbonate buffer is preferred. Glutathione (not present in the oocyte retrieval and handling medium) is included, to assist in the process of sperm head decondensation. Alanyl-glutamine (present in the oocyteretrieval and handling medium) is omitted from the present medium because it can impair sperm function and reduce fertilization. The same is true of the chelating agent EDTA, which (as will be discussed later) is present in the embryo development media. HSA, the most abundant macromolecule in the Fallopian tube and uterus, is included to support sperm and embryo function. Hyaluronate, which promotes sperm motility, and works in tandem with HSA, is also included. Because sperm tends to contain high levels of bacteria, one or more antibiotic substances are also included Penicillin, streptomycin, and/or gentamycin are preferred antibiotics. Table 3 sets out. the preferred amounts and ranges for these various components.

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4. The ICSI Medium

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In circumstances where it is desired to accomplish fertilization by other than natural interaction of sperm and oocyte, such as where the sperm is unable to fertilize the oocyte due to a thickened zona pellucida surrounding the oocyte, or where the sperm is from a male-factor patient, the sperm may be transported into the oocyte by a technique called intracytoplasmic sperm injection (ICSI). When the ICSI technique is used, the cumulus cells are removed from the oocyte, and sperm is injected into the oocyte's interior using a glass pipette. The present invention contemplates use of a single medium to bathe the oocyte and also to serve as a carrier for the sperm as it is transported by injection into the oocyte. The medium, accordingly, is preferably whighly compatible with the interior and exterior of the oocyte. The ionic constituents

in the preferred medium are similar to those found in the oocyte retrieval and handling medium, except that phosphate is omitted, to avoid metabolic and homeostatic stress, and the magnesium-to-calcium ratio is 2:1. This ratio of magnesium to calcium is felt to be highly beneficial to the oocyte. Because ICSI is a clinical procedure performed outside the incubator, a buffering system that is effective in a normal atmosphere is used. MOPS and HEPES are accordingly preferred buffers for this medium. Because the cumulus cells have been removed from the oocyte, and the sperm is at the conclusion of its independent life, glucose, the main energy source for cumulus cells and sperm (but not the oocyte) is omitted from the medium. Pyruvate and lactate levels are increased, as these are a primary energy source for the oocyte. Only the non-essential amino acids most abundant in the oocyte - glycine, proline, serine and taurine - and glutamine (in the stable form alanyl-glutamine) are retained in the medium to avoid osmotic and pH stress and to nourish the oocyte. Preferably, the ICSI medium also includes hyaluronate or polyvinylpyrollidone (PVP), to immobilize or slow the sperm so that they may be captured in the ICSI pipette. Table 4 sets out the preferred amounts and the ranges of these components in the ICSI medium. Moreover, an alternative formulation of the ICSI medium includes hyaluronidase, which alternative formulation is used to pretreat the oocyte, to break down the hyaluronate gel holding the cumulus cells around the oocyte. This medium is referred to above as denuding medium, and lacks hyaluronate and PVP but includes hyaluronidase. The composition of the denuding medium includes the constituents of the ICSI medium (except hyaluronate and PVP) in the preferred amounts and ranges shown in Table 4 plus hyaluronidase in a preferred about of 40 IU/ml and a preferred range of Oct-80. Optionally, HSA may be included in the denuding medium in the preferred amount of 5mM and the preferred range of 1.0 - 10mM.

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	general to the second of bound on Table 4 and on nation a brace special of
	Component
	Most Preferred Supplied Preferred
	Concentrati n o lai de ma Range, e
	NaCl 90.08 no 2000 1 75.0 2 105
5	KCI 5.5
	MgSO ₄ 7/H ₂ O ₅ 2.0,4 - 4 NaHCO ₃ 5.20,-10,
	NaHCO ₃ MOPS / HEPES 1
	the second secon
•	CaCl ₂ .2H ₂ O. 1 O.5 - 2.0 O.5 - 2.0 O.5
10	NaLactate (L-isomer) a fight of property to the first 10.5 from which others in 5.0 = 20 mg.
10	NaPyruvate 0.32 Only the second of the sec
	The second of th
	The form of the control of the second of the
	Alanyl - Glutamine 0.5 0.1 - 2.0 Glycine 0.5 0.5 0.1 - 2.0
15	Proline Serine 0.1 0.05 - 2.0 0.05 - 2.0
	Taurine 100 100 100 100 100 100 100 100 100 10
	HSA to a second to the second
	Hyaluronate PVP 10% 1-20% 1-20% 10 The property of the proper
20	* Concentrations are in millimoles unless otherwise indicated; the medium is aqueous. The medium is aqueous. The medium is a property of the m
	aqueous. Take them are larged the setted (3 of the separately display and a result of
	5. Embryonic Development Medium G1.2.
	5. Embryonic Development Medium G1.2. The present invention includes an embryonic development medium G1.2. The preferred application of this medium is to support development of the early one-to-
	preferred application of this medium is to support development of the early one-to-
25	eight cell embryo. As depicted in Table 5, the preferred medium has a backbone of
	ionic constituents and non-essential amino acids that is similar to that found in the
	oocyte retrieval and handling medium. Unlike the oocyte retrieval and handling
	medium, the G1.2 medium contains the component EDTA, which supports embryonic
	development and is believed to bind and disable toxins that might have a deleterious
30	effect on the early embryo, and which also suppresses glycolysis. In addition, this

medium includes HSA and hyaluronate, in concentrations that are thought to support early embryonic development.

The preferred formulation of medium G1.2 differs from the previously published medium G1 in several important respects. First, research has shown that an 5 elevated phosphate concentration may not provide optimal conditions for growth of the developing embryo. Accordingly, the phosphate concentration has been decreased. Second, hyaluronate has been added to work in tandem with HSA. Third, alanyl-glutamine has been substituted for glutamine. A significant problem for embryo culture with amino acids is the natural decomposition of amino acids to ammonium, which decomposition is accelerated at higher temperatures such as the physiological temperature (37 degrees Celsius) used in IVF procedures. Ammonium can be toxic to embryos. Moreover, glutamine is especially prone to decomposition to ammonium within solution. Since embryos are generally cultured in medium GI or G1.2 for an extended period of up to about 48 hours, a significant quantity of ammonium can develop in the medium and be a significant inhibitor to embryo 15₀ development. Accordingly, the use of alanyl-glutamine provides substantial advantages; it is a particularly stable form of glutamine and is not prone to breaking down in solution. Also, the concentration of alanyl-glutamine in G1.2 has been reduced to .5 mM. These three modifications make G1.2 a significantly improved 20 medium for early embryonic development over medium G1. The most preferred amounts and preferred ranges of the components of medium G1.2 are depicted in Table 5.

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	Comp nent			A	<u>G1.2*</u> □ \ū. \vvoi. ≥ s	क्ट्राट अ	O R
	to a straight	इसी का भर्ग दल	90% N 200 12	Most Prefer	redat barroter	ឲ្យ ១៩៤៣	Preferred
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5	NaCl	Market A. D.	alan waa i	90.08	ដាចបានសម	ections.	80.0 - 100
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	MgSO ₄ .7H ₂ O	medera di	Array Dave	10 37. 1 says	arris și Lent.	K - h. f	0.2 - 2.0
	เปลี้ คยได้เลก	ar Timests	e Himself by	n' bo Pilar	e ned trilleri	Bertit .	13.0 - 30
	$CaCl_{2,2}H_{2}O_{\mathrm{proj},\mathrm{la}}$						
10	Glucose ad and	ំខ្មុំ យ៉ាង	aligned in Landam .	12 10 12 015 11 15	tyrransk fil di	ស្រាល់ ស	0.05 -5.0
	NaLactate (L-						
	NaPyruvate	ar Mag 1673	e is excess (see as	0.32	***		0.1 - 1.0
	edit to junar in	. જે. ાતું પ્∷ેલ્	ice no a sem	at de annom	the contractions	a Verterio	\$C 15 - 1
	Alanine Asparate	adia- (19 19 g p (1) 30 ()	days 94 —	cardor sirbus:	យោធិន	0.01 - 0.5
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	ni bathi nu ca	n Alexandra	geo i Solviti sur	क्षांत्र और एक्सक	reitried rauge	n derne ede	Taronta _i)
	HSA Hyaluronate	÷		5mg/ml			1 - 10.0
	Tryaturonate			0.1 mg/ml			0.02 - 0.5

* Concentrations are in millimoles unless otherwise indicated; the medium is aqueous.

6. Embryonic Development Medium G2.2

Medium G2.2 is also formulated to support embryonic development. Its preferred use is with embryos from the eight-cell to the blastocyst stage (around 100 cells) to around one-hundred cell stage. The backbone of ionic constituents and non-essential amino acids preferably found in medium G2.2 is essentially the same as used with medium G1.2, except that the concentration of alanyl-glutamine has been increased. This reduces the risk of subjecting the embryo to osmotic stress as it is

moved from medium G1.2 to medium G2.2. Taurine is omitted because its benefits to the embryo appear to be confined to the period prior to compaction. Glucose, lactate and pyruvate are included as carbohydrates, except that the concentration of glucose is increased, while lactate and pyruvate are decreased, as compared to medium G1.2.

5 This modification in carbohydrate levels is in response to the increasing ability of the developing embryo to metabolize glucose as an energy source, and reflects also the observed composition of the female reproductive tract. Eagle's essential amino acids are included in medium G2.2 because they are necessary to stimulate the growth of the inner-cell mass of the blastocyst. Vitamins are added as a group because in

10000 animal studies they tend to facilitate the function of the blastocyst, including fluid accumulation in the cavity of the blastocyst. Importantly, this medium lacks EDTA. The preferred amounts and ranges of the components of medium G2.2 are depicted in Table 6. ic yet!

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	Component		<u>A</u>	P. das - das -	50 1501 <u>B</u>
			Most Preferre	<u>•d</u>	Preferred
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	be consider	ឌី ខេទ្ត នេះនាំមេ			Range
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	T/CI	Bapta Mild for a read .			
			0.20		(0.00) - (0.0)
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	NaHCO3	राज्य संदर्भ तकार ^प ्रिस	25	n' sa serie e de l'are	
	Glucose January	nistemoth: alt a ga	oti # 6:15.00 /	di cukere inci tadi.	100 005 55
	NaLactate (L-ison	ntholholes, and a ga per) gest bed before bed	.5.87	and a second of the second of the	2.0 - 20.0
25	NaPyruvate ****	Syst due naouer sau	0.1	me management	0.01 - 1.0
	Alanine	n decreed in Table 7.	# B 1 3 0.1 1 18	dogdaeal kor	0.01 - 0.5
	Asparate		0.1		0.01 - 0.5
	Asparagine		0.1		0.01 - 0.5
	Glutamate.	· "如此"	0.1		0.01 - 0.5
30	Alanyl - Glutamine	way na marana a	5.13 (42.1 <u>)</u> 13 1	1	0.01 - 2.0
	Glycine		0.1		0.01 - 0.5
	Proline	1. 2. 21. 1	0.1		0.01 - 0.5
	Serine	471 1 2 W 1	0.1	•	0.01 - 0.5
	L-Arginine-HCl		0.6		0.1 - 1.2
35	() L-Cystine 2HCl	31° (°)	0.1		0.05 - 0.25

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9	L-Histidine-HCI-H2O	err - F and 0.2 E / D mails	^{(m, g', M, o} 0.1 - 0.4
	L-Isoleucine	0.4	0.1 - 0.8
•	L-Isoleucine L-Leucine	0.4	0.1 - 0.8
	L-Leucine L-Lysine-HCl	ones lauto 19 0.4 %, on belonishing o	0.1 - 0.8
5	L-Methionine	Q.1 was born atomat said	0.05 - 0.25
	L-Methionine L-Phenylalanine	0.2	0.1 - 0.4
	L-Threonine and the state of the state of	्याः । अर्थे संघान्य 0.4एति स्वित्योद्धः । । य अस्य	8.0 - 1.0 Property of the state
	L-Tryptophan L-Tyrosine 2Na	0.5 pole our many party	0.1 - 0.9
	L-Tyrosine 2Na	0.2	0.1 - 0.4
10	L-Waline of the Brander story or the	or the order of the contraction	0.12 0.8
	D-Ca Pantothenate Choline Chloride	0.002 CO CHARTA	9.22. 0.001 - 0.004
	Choline Chloride	0.007	0.003 - 0.01
	Folic Acid was the wag a rechebble of	motion of the model of the scene	0.001 - 0.0045
	i-Inositol Niacinamide	was a second of the following graft to	0.005 - 0.02
15	Niacinamide Annual Annu	0.0082	0.004 - 0.016
	Pyridoxal HCl - Attantiant with the bearing	皮膚性a、560.0049年 (2.21年2月 年) (4.	0.002 - 0.01
	Riboflavin Thiamine HCl	0.0003	0.0001; - 0.0006
	Thiamine HCl	0.003	0.001 - 0.006
	HSA	5mg/ml	
20	Hyaluronate	0.1mg/ml	0.02 - 0.5

^{*} Concentrations are in millimoles unless otherwise indicated; the medium is aqueous.

7. **Embryo Transfer Medium**

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*(*1)

Component

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The preferred embryo transfer medium contains the same formulation of

constituents as medium G2.2 except that a much higher concentration of hyaluronate is included. In the human reproductive system, research indicates that there is a caused receptor on the embryo for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and that there is also a receptor for hyaluronate and hyalu hyaluronate on the endometrium of the mother. Hyaluronate is thought to act like and biological glue that assists the embryo in binding to the endometrium and, 30 accordingly, supports implantation. The preferred amount and ranges of the accordingly supports implantation.

constituents of the embryo transfer medium are depicted in Table 7. Asparata

		Table 7	ote wind D
<u>C</u>	Composition o	f Embryo Transfer Medium	grade Wrate
		<u>A</u>	<u>B</u> °
	4.5	Most Preferred	<u>Preferi</u>

Concentration

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Asperaging

Preferred

Range

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35	NaCi	3 3	90.08	13475 s 680.0 - 100

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	KCl hase ford as well and I w	Constant of the Parker of Alberta	2.5.5.5
	NaH2PO4.2H2O	vi (#+#hr.) sh.x 0:25 wey (-) -1	3.3 - 7.5
	MgSU4./H2()	-	0.05 1.5
_	NaHCO3h E verbil ko arego mil	lanca Compine <mark>d</mark> Follows	0.2 - 4.0
5	CaCl2.2H2O	. Some winer skiller 8 hereite og e	15 - 30.0
	Glucose	3.15	0.0 - 2.0
	NaLactate (L-isomer)	5.87	0.5 - 5.5
	NaPyruvate	0.1	2.0 - 20.0
10	Alanine	0.1	0.01 - 1.0
10	Asparate Sandalastic		0.01 - 0.5 0.01 - 0.5
	Asparagine	0.1	0.01 - 0.5
7,934	Glutamate	o.1	0.01 - 0.5
	Alanyl - Glutamine	1 1	0.01 - 0.3
1.5	Glycine	0.1	0.01 - 2.0
13	Proline (a) En	0.1	0.01 - 0.5
	Serine	0.1	0.01 - 0.5
	L-Arginine-HC1	0.6	0.1 - 1.2
	L-Cystine 2HCl	0.1	0.05 - 0.25
20	L-Histidine-HCl-H2O	0.2	0.05 = 0.25
20	. 2.10010401110	0.4	0.1 - 0.8
	L-Leucine	0.4	0.1 - 0.8
	L-Lysine-HCl	0.4	0.1 - 0.8
	L-Methionine	0.1	0.05 - 0.25
25	L-Phenylalanine L-Threonine	0.2	0.1 - 0.4
23	L-Tryptophan	0.4	0.1 - 0.8
	L-Tyrosine 2Na	0.5	0.1 - 0.9
	C . T 37-11	0.2	0.1 - 0.4
	D-Ca Pantothenate	0.4	0.1 - 0.8
30	Choline Chloride	0.002	0.001 - 0.004
	TO - 1' A	0.007	0.003 - 0.01
	i-Inositol	0.0023	0.001 - 0.0045
;	Niacinamide SEG	0.0111	0.005 - 0.02
	Pyridoxal HCl	0.0082	0.004 - 0.016
35	Riboflavin	0.0049	0.002 - 0.01
	Thiamine HCl (mg/m)	0.0003	0.0001 - 0.0006
	and the second of the second of the	0.003	0.001 - 0.006
	Hyaluronate	0.25mg/ml	0.05 - 1.0
	*Concentrations are in millimoles	cipolitic Office from the configuration of the conf	

*Concentrations are in millimoles, unless otherwise indicated; the medium is aqueous, and the medium is a concentration are in millimoles, unless otherwise indicated; the medium is a queous, and the medium is a concentration of the medium is a concentration.

8. <u>Cryopreservation Medium</u>

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The present invention involves a separate medium to be used in cryopreservation of the oocyte and embryo. The preferred formulation to be used includes ionic constituents and a buffer, preferably MOPS or HEPES, as well as the

carbohydrates lactate, pyruvate and glucose. Optionally, HSA may be included. In addition, the medium may include certain additives, glycerol, ethylene glycol, DMSO, propanedial, and/or sucrose. The preferred amounts and ranges of the constituents of the cryopreservation medium are depicted in Table 8.

Table 8

<u>Composition of Cryopreservation Medium*</u>

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Component	1 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2	<u>A</u>	. The street of B
$\frac{1}{2} \frac{\partial}{\partial x} \frac{\partial}{\partial x} = \frac{1}{2} \frac{\partial}{\partial x} \frac{\partial}{\partial$		Most Preferre Concentration	
NaCl	1.3 1.3	90.08	75.0 - 105
KCI	1.0	5.5	5076 1034 Surgay 3.5 - 7.5
10 MgSO ₄ , 7H ₂ O). 1 	2	13.7% 2011 0.4 - 4
Na2PO4.2H2O		0.25	Set 10 Report by Set 1.5 (1) on the set of t
NaHCO ₃		5	19) see no 11. 1 2.0 - 10
MOPS/HEPES	£ £	20	administrackled contact 10±25.0 cathanger Test
CaCl ₂ .2H ₂ O		1	ลดีร์ อกเรอารูไรน์ ระยั !.0.5 -i 2.0 อยะเทษทรร์ อ3-G
15 NaLactate (L-isomer)	\$ 99.0 ***** ************	5.87	tobacted saltered by block 2.0520
NaPyruvate	27 '0, (0.32	SolvaniQ.1 (m) 1.0
Glucose	900000 10000	1	Dir is 60.5% 5.5
HSA	E00. 0	5mg/ml	作れる品面:0年10

ADDITIVES

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Glycerol and/or ethylene glycol and/or DMSO and/or propanedial and/or sucrose
Range for all except sucrose is 2 to 20%; range for sucrose is 0.1 to 1M (2005) 1. 10 (2005)
Concentrations are in millimeters unless otherwise indicated; the medium is aqueous 2005)

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B. Sequential Culture Media Process

Instead of immersing human reproductive cells in a single culture medium

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25 throughout the various procedures used in IVF, the present invention involves a

process by which the reproductive cells may be moved through a sequence of distinct culture media as the various IVF procedures are carried out. In one aspect of the invention, the culture media are specifically formulated to provide a physical environment similar to that found within the female reproductive tract and conducive to growth and development of human reproductive cells during various stages of the IVF process. In a further aspect of the invention, the specifically formulated culture media can be applied to support the reproductive cells in one or more of the following procedures: oocyte retrieval and handling; oocyte maturation; ordinary fertilization; oocyte, zygote and embryô examination and biopsy; embryonic development to the eight-cell stage; embryonic development to the blastocyst stage; embryo transfer; and cryopreservation. Most preferably, the media will be applied sequentially during each of the applicable stages of the IVF process to which the media have been adapted. It should be noted that there is significant variation among clinics and laboratories as to equipment and specific procedures used to accomplish each of the principal steps in the IVF process. The present invention contemplates that the sequential media and process described herein may be utilized and/or readily adapted for use with the wide variety of equipment and procedures employed in IVF practice. What follows is a more detailed discussion of exemplary applications of the media during IVF and related methodology: as accessor to the second .} in the color of the artificial of the parameter and or a mile creamer.

1. Coli Oocyte Retrieval and Handling; Embryo Handling

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Referring to Fig. 1, an initial procedure in the illustrated IVF process 100 is oocyte removal or retrieval (102) from the mother's ovary. This is typically performed vaginally using a fine needle attached to and guided by a transvaginal ultrasound probe. The needle is ordinarily connected to fine Teflon tubing and thence to an aspiration regulator controlled by a vacuum regulator. The aspirate is collected in test tubes or other appropriate vessels, containing medium. The medium may be used to preliminarily wash the needle and tubing, and other equipment used in the procedure. In some clinical settings, the medium may also be used with a specially adapted needle to flush the follicle and aid in removal of the oocyte. The medium, equipment used, and aspirate are maintained, so far as possible, at 37 degrees Celsius. If a bicarbonate buffer system is used in the medium, the procedure ordinarily is

atmosphere. In the absence of such atmospheric controls, the medium must contain a MOPS or HEPES buffering system.

The illustrated process 100 present invention contemplates that the oocyte retrieval and handling medium may be used in each phase of the retrieval process. The process of using the oocyte retrieval and handling medium may involve washing any equipment that may come into contact with the oocyte during removal from the ovary, and that may be used to aspirate, flush and/or wash the oocyte during the removal and collection process. Following removal from the ovary, the oocyte may be washed with medium. Optionally, the oocyte may be stored in the medium for a period.

In addition, it is contemplated that the medium may be used during other clinical or laboratory procedures where the oocyte is manipulated or handled, and also in procedures where the embryo is manipulated or handled, especially where these occur outside the isolette. Examples would include examination of the oocyte following retrieval from the mother, examination of the oocyte following the fertilization step, and examination of the embryo to determine whether it has developed the eight-cell stage. In each of these examples, the oocyte/embryo would be bathed in the medium as it is withdrawn by pipette from the culture dish or test tube, and would remain immersed in the medium while examined under a microscope or with other equipment. The illustrated implementation of the invention also contemplates that an alternative formulation of this medium, which is calcium and magnesium free, may be used during biopsy procedures.

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In the event the collected oocytes are immature, the illustrated process 100 envisions that a second medium may be used to support and promote development of the oocyte during maturation (106). The oocyte maturation medium would ordinarily be used to treat and mature the oocyte following a collection procedure, in which the oocyte is retrieved from the ovary using oocyte retrieval and handling medium. The retrieval and handling medium and maturation medium have a very similar backbone of ionic constituents and amino acids and glutamine, such that as the oocyte is moved

from one medium to another it experiences minimal ionic shock. The illustrated process 100 includes immersing the oocyte and surrounding cumulus cells in the maturation medium for a period of about 30-48 hours, or until the oocyte is mature. The illustrated process 100 then contemplates removing the oocyte from the maturation medium and immersing it in either sperm preparation and fertilization medium or ICSI medium for purposes of fertilization.

In accordance with the invention, the oocyte maturation medium may be applied to the oocyte retrieval process (102), in place of the oocyte retrieval and handling medium described herein. Additionally, a conventional culture medium, such as Ham's F-10 or medium TCM-199 with or without a HEPES buffer, may be employed for immature oocyte retrieval and handling, before immersion of the oocyte in the maturation medium of the present invention. Once maturation is complete, the oocyte will be immersed in a medium for ordinary IVF fertilization procedure (110), or will be immersed in an ICSI medium in preparation for assisted insemination through an ICSI procedure (112).

3. Sperm Preparation and Fertilization

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The illustrated process 100 contemplates that the collected oocytes will ordinarily be washed and immersed in, and allowed a period of pre-incubation culture within, a first portion of the sperm preparation and fertilization medium. This period of pre-incubation culture (104) may last up to about six (6) hours. Oocytes permitted a period of pre-incubation culture typically have higher fertilization rates.

The process 100 also contemplates that the sperm may be separately washed and stored in a second portion of the sperm preparation and fertilization medium to purge it of bacteria and any other contaminants that may be present. Sperm preparation (108) may involve dilution of semen with the medium, centrifugation, and resuspension of the concentrated sperm in a new portion of medium. In the "swim up" method of sperm preparation, the medium containing sperm is centrifuged, the medium is drained off, and a new portion of medium is poured over the spundown sperm pellet. The sperm is given a period to "swim up" into the fresh medium. That layer of fresh medium, containing the more motile sperm, is then poured off and centrifuged, and the process is repeated. In another aspect of the invention, the sperm

preparation and fertilization medium may be used in one or more gradient separation procedures, such as the Percoll procedure. The present invention envisions that the sperm preparation and fertilization medium may be used as the medium in any of the sperm preparation procedures that may be used for IVF. 30.001

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Once the sperm is prepared (108), the sperm is then examined and counted while in medium, and a desired quantity is added to the portion of medium which contains the oocyte. The sperm and oocyte are permitted to remain together in the medium for a period of up to several hours, and, in some laboratories, for a much longer period, as long as about sixteen (16) to eighteen (18) hours. The invention further contemplates that, following a period of immersion in the medium with sperm, the oocytes will be removed and examined (114) to determine whether fertilization (110) has occurred. When removed for examination, the oocytes will continue to be bathed in the sperm preparation and fertilization medium if the examination can be conducted in an isolette. If not, then, as noted above, the oocyte retrieval and handling medium may be used for handling and examination of the oocytes.

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4. Fertilization by Direct Injection of Sperm into the Oocyte (ICSI & Technique)

In the ICSI process (112), sperm may be directly injected into the cytoplasm of the oocyte through a very fine pipette or needle. The process 100 contemplates washing the sperm with a portion of the ICSI medium containing hyaluronate and/or PVP, and then placing the sperm in the medium. The process 100 further involves drawing a microvolume of the medium containing sperm into the pipette and then injecting the medium and sperm into the interior of the oocyte as process and broads and broads.

The illustrated process 100 further contemplates that the obcyte may be bathed in another portion of the ICSI medium during the ICSI process. An alternative design formulation of the ICSI medium may be used, supplemented with hyaluronidase, for denuding pretreatment (105) of the oocyte prior to the ICSI process. Pretreatment involves immersing the oocyte in the ICSI medium supplemented with hyaluronidase for a period until the oocyte becomes denuded of all or most of its surrounding cumulus cells. Following pretreatment, the oocyte is injected with sperm carried in a separate portion of medium, using an ICSI pipette, as provided above.

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After the ICSI injection process (112) is complete, it may be necessary to examine (114) the oocyte to evaluate whether fertilization has been effective and the oocyte is intact and healthy. Examination may occur in the ICSI medium bathing the oocyte, or may occur in the oocyte retrieval and handling medium as described above.

5. Embryonic Development to Eight-Cell Stage

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Medium G1.2 is applied to the early embryo, following formation of the zygote. After the zygote is identified, it is washed with medium G1.2, and then immersed in G1.2 medium for a culturing period (116) of up to about forty-eight hours. During this time the embryo undergoes development from the one-cell to around the eight-cell stage, and is removed at about the eight-cell stage. Examination (118) of the embryo may occur in the G1.2 medium, or in the oocyte retrieval and handling medium, as described above.

6. <u>Embryonic Development to Blastocyst Stage</u>

The illustrated process 100 contemplates that medium G2.2 will be used to

15 culture (120) the developing embryo to the blastocyst stage, preferably from about the
eight-cell stage to about the one-hundred-cell stage. The process 100 also
contemplates that, once the embryo reaches the blastocyst stage, and assuming that
the embryo is judged on examination (124) to be viable, it is removed from the G2.2
medium and prepared for transfer into the uterus. In some laboratories, the G2.2

20 medium may, optionally, be used for embryo transfer as well. Examination (124) of
the embryo may occur in the G2.2 medium or in the oocyte retrieval and handling
medium, as described above.

7. Embryo Transfer

The process 100 contemplates that the embryo transfer medium will serve as a carrier for the embryo as it is transferred (126) back into the mother. The embryo will be bathed in the transfer medium, the medium containing the embryo will be drawn into the transfer catheter, the catheter will be inserted into the mother's uterus, guided by an ultrasound probe, and the medium containing the embryo will be injected into the uterus.

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8. Cryopreservation of (Str.) serves to complete the page 14.

The cryopreservation medium may be used for storing, freezing, thawing, witrification, and warming the oocyte, prior to fertilization. The same medium may be used for storing, freezing, thawing, vitrification, and warming the cleavage stage embryo, as well as the embryo in the eight to one hundred cell stage.

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While the present invention has been described in relation to one embodiment, it will be appreciated that the invention may be utilized in numerous additional embodiments and procedures. Such additional embodiments and procedures are within the scope of the present invention, as defined by the claims which follow.

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A method for use in an IVF process, wherein the process involves some or all of the stages of: oocyte retrieval and handling; oocyte maturation; sperm preparation; fertilization; oocyte, zygote and embryo examination and biopsy; embryo development; embryo transfer; and cryopreservation said method comprising the steps of:

supporting reproductive cells in a first support medium during a first stage of said stages, said first support medium including a core group of salts; and

supporting reproductive cells in a second support medium different than said first support medium during a second stage of said stages, said second support medium including substantially said same core group of salts as said first support medium, said core group of salts utilized in both of said first and second support media thereby minimizing any stress and trauma to reproductive cells incident to transfer between the first and second support media;

wherein no more than one of said first and second stages is one of said embryo development stage and said embryo transfer stage.

- 2. A method as set forth in Claim 1, wherein said first stage is one of embryo examination and oocyte retrieval and handling.
- 3. A method as set forth in Claim 2, wherein said first support medium comprises water, ionic constituents and a buffer.
- Amothed as set forth in Claim 2, wherein said first support medium comprises one of 4-Morpholinepropanesulfonic acid (MOPS), N-2-hydroxyethylpiparazine-Nil 2-ethane sulphonic acid (HEPES) or bicarbonate.
- 5. A method as set forth in Claim 2, wherein said first support medium compa ses carbohydrates; bit a minochia as a transfer of the compa ses carbohydrates; bit a minochia as a transfer of the compa ses carbohydrates; bit a minochia as a transfer of the compa ses carbohydrates; bit a minochia as a transfer of the compa ses carbohydrates; bit a minochia as a transfer of the compa ses carbohydrates; bit a minochia as a transfer of the compa ses carbohydrates; bit a minochia as a transfer of the compa ses carbohydrates; bit a minochia as a transfer of the compa ses carbohydrates; bit a minochia as a transfer of the compa ses carbohydrates; bit a minochia as a transfer of the compa ses carbohydrates; bit a minochia as a transfer of the compa ses carbohydrates; bit as minochia as a transfer of the compa ses carbohydrates; bit as minochia as a transfer of the compa ses carbohydrates; bit as minochia as a transfer of the compa ses carbohydrates; bit as minochia as a transfer of the compa ses carbohydrates; bit as minochia as a transfer of the compa ses carbohydrates; bit as minochia as a transfer of the compa ses carbohydrates; bit as minochia as a transfer of the compa ses carbohydrates; bit as minochia as a transfer of the compa ses carbohydrates; bit as minochia as a transfer of the compa ses carbohydrates; bit as a transfer of the compa ses carbohydrates; bit as a transfer of the compa ses carbohydrates; bit as a transfer of the compa ses carbohydrates; bit as a transfer of the compa ses carbohydrates; bit as a transfer of the compa ses carbohydrates; bit as a transfer of the compa ses carbohydrates; bit as a transfer of the compa ses carbohydrates; bit as a transfer of the compa ses carbohydrates; but a transfer of the compa ses carbohy
- 6. A method as ser forth in Claim 2, wherein said first support medium comprises non-essential amino acids.
- 7. A method as set forth in Claim 2, wherein said first support medium comprises glutamine and singular to the comprise glutamine and singular to the comprise glutamine.
- 8. A method as set forth in Claim 2, wherein said first support medium comprises antibiotics.

9. A method as set forth in Claim 1, wherein said first support medium is free from calcium and magnesium and said first support medium is used in biopsy procedures.

10. A method as set forth in Claim 1, wherein said first stage comprises to cocyte maturation.

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- 11. A method as set forth in Claim 10, wherein said step of supporting reproductive cells in a first support medium comprises supporting an oocyte in said first support medium for a time period following oocyte collection to promote gate and development prior to fertilization.
- 12. A method as set forth in Claim 10, wherein said first support medium is comprises magnesium and calcium disbursed in an aqueous solution.

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- 14. A method as set forth in Claim 10, wherein said first support medium comprises one or more growth factors such as insulin transferin selenium (ITS); and insulin-like growth factor (IGF), and epidermal growth factor (EGF).
- 15. A method as set forth in Claim 10, wherein said first support medium force comprises one or more hormones such as follicle stimulating hormone (ESH) and human chorionic gonadotrophin (hCG).
- 16. A method as set forth in Claim 1, wherein said first stage comprises one of sperm preparation and fertilization. The stage comparation and fertilization.
- 17. A method as set forth in Claim 16, wherein said first support medium that comprises carbohydrates.
- 18. A method as set forth in Claim 16, wherein said first support mediting comprises one or more of bicarbonate, glutathione, HSA and hydronate.
- 19. A method as set forth in Claim 16, wherein said first support medium a comprises antibiotics.
- 20. A method as set forth in Claim 16, wherein said first support medium and comprises nonessential amino acids.
- 21. A method as set forth in Claim 16, wherein said first support medium with is free of EDTA.

22. 30 A method as set forth in Claim 1, wherein said first stage comprises oocyte retrieval and handling and said second stage comprises one of sperm preparation and fertilization.

23. A method as set forth in Claim 22, wherein said second support medium has an elevated concentration of sodium as compared to said first support medium.

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- 24. A method as set forth in Claim 22, wherein said second support medium has an elevated concentration of phosphate as compared to said first support medium.
- 10 25. A method as set forth in Claim 1, wherein said first stage utilizing said first support medium is part of a process of intracytoplasmic sperm injection (ICSI).
 - 26. A method as set forth in Claim 25, wherein said ICSI process comprises removing cumulus cells from an oocyte, incubating sperm, and injecting the sperm into said oocyte; and

into said second

support medium

- 27. A method as set forth in Claim 25, wherein said first support medium used in said ICSI process is free from phosphate.
- 28. A method as set forth in Claim 25, wherein said first support medium used in said ICSI process comprises one of MOPS process, HEPES and bicarbonate.
 - used in said ICSI process comprises carbohydrates.
- used in said ICSI process is free of glucose.
- used in said ICSI process comprises non-essential amino acids.
- 32. 5. A method as set forth in Claim 25, wherein said first support medium used in said ICSI process comprises glutamine.

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30 A method as set forth in Claim 26, wherein said first support medium is used for supporting said sperm as part of said ICSI process and comprises one of hyaluronate or polyvinylpyrolidorie (PVP).

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34. A method as set forth in Claim 25, wherein said first support medium comprises magnesium and calcium in an aqueous solution. He is the action of the state of

- 35. A method as set forth in Claim 25, wherein said first stage comprises denuding an oocyte and said first support medium comprises hyaluronidase.
- 36. A method as set forth in Claim 1, wherein said first stage comprises to embryonic development.

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- 37. A method as set forth in Claim 36, wherein said step of supporting reproductive cells in a first support medium comprises supporting a zygote in said first support medium for a time period that is one of at least 48 hours or through at least the eight-cell stage.
- 38. A method as set forth in Claim 36, wherein said first support medium? comprises carbohydrates.

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- 39. A method as set forth in Claim 36, wherein said first support medium of comprises non-essential amino acids.
- 40. A method as set forth in Claim 36, wherein said first support medium comprises one or more of HSA, and hyaluronate.
- 41. A method as set forth in Claim 36, wherein said first support medium comprises glutamine.
- 42. A method as set forth in Claim 41, wherein said glutamine comprises alanyl-glutamine.
- 43. A method as set forth in Claim 1, further comprising the step of supporting reproductive cells in a third support medium different than said first and second support mediums during a third stage of said stages on 2000 of 1200 bits of the second support mediums during a third stage of said stages on 2000 of 1200 bits of the second support mediums during a third stage of said stages.
- 44. A method as set forth in Claim 43, wherein both said second stage and stage comprise embryo development and transfer. at 1500 to the contract to the contr
- 45. A method as set forth in Claim 43, wherein said third support medium is used subsequent to said second support medium and said third support medium has an adepressed concentration of one of lactate and pyruvate relative to said second medium.
- is used subsequent to said second support medium and said third support medium an elevated concentration of glucose relative to said second support medium, specially as a second support medium.

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47. A method as set forth in Claim 36, wherein said step of supporting reproductive cells in a first support medium comprises supporting an embryo in said first support medium for a time period that is one of from about 48 to 96 hours and from about the eight-cell stage to about the one hundred cell stage.

48. A method as set forth in Claim 36, wherein said first support medium comprises non-essential amino acids and is free from taurine.

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- 49. A method as set forth in Claim 36, wherein said first support medium comprises essential amino acids.
- 50. A method as set forth in Claim 36, wherein said first support medium 10 comprises vitamins.
 - 51. A method as set forth in Claim 30, wherein said first support medium comprises HSA.
 - 52. A method as set forth in Claim 36, wherein said first support medium is free from EDTA.
- 15 53. A method as set forth in Claim 36, wherein hyaluronate is added to said first support medium for embryo transfer.
 - 54. A method as set forth in Claim 1, wherein said first stage comprises cryopreservation.
- 55. A method as set forth in Claim 54, wherein said first support medium 20 comprises one of MOPS or HEPES.
 - 56. A method as set forth in Claim 54, wherein said first support medium comprises carbohydrates:
 - 57. A method as set forth in Claim 54, wherein said first support medium comprises HSA.
- 25 58. A method as set forth in Claim 54, wherein said first support medium comprises one or more of glycerol, ethylene glycol, DMSO, propanediol and sucrose.
 - 59. A method as set forth in Claim 36, wherein said first support medium comprises EDTA.
- 60. A method as set forth in Claim 54, wherein said first support medium comprises nonessential amino acids.

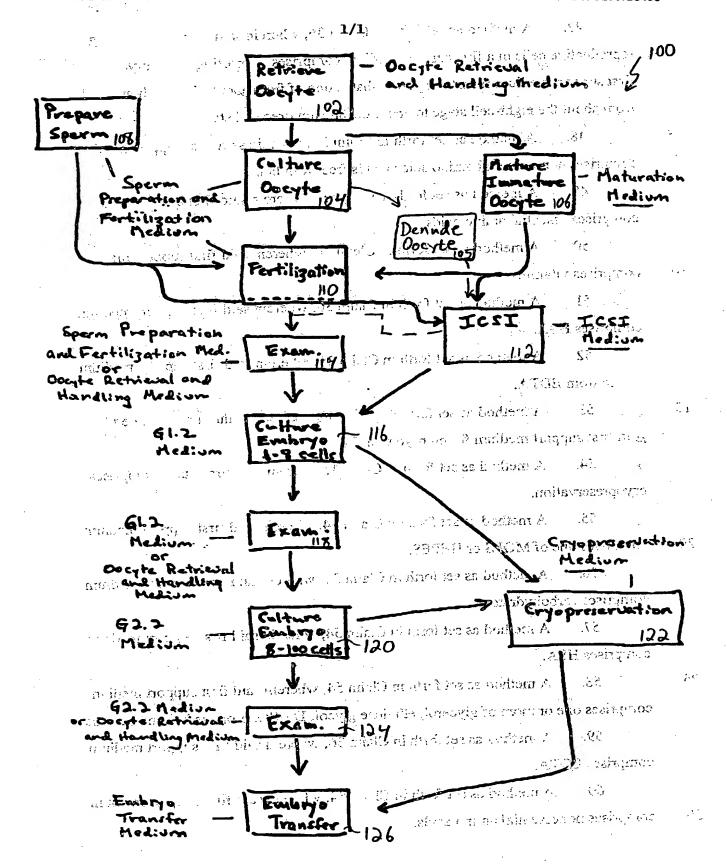


Figure 1

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INTERNATIONAL SEARCH REPORT

International application No. PCT/US99/28408

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/28408

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)	er en
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INTERNATIONAL SEARCH REPORT

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International application No. PCT/US99/28408

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BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING This ISA found multiple inventions as follows: IN THE HALL OF HE CHARLES

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as ON A SECTION AND A SECTION ASSESSMENT AS A SECTION AS A S

First, each stage claimed in the method is a distinct species, such as the species of the stage of claim 2, the species of the stage of claim 10, of claim 16, of claim 22; of claim 25, of claim 36, of claim 43, and the stage of claim 54. Further, each medium is a distinct species. For example, the species of the stage of claim 2 has distinct species of media from one of claims 3-9 (7 species). The species of the stage of claim 10 has the media of the species of claims 12-15 (4 species). The species of the stage of claim 16 has the species of the media of claims 17-21 (5 species). The species of the stage of claim 22 has the media of the species of claims 23 and 24 (2 species). The species of the stage of claim 25, has the media of the species of claims 27-35 (9 species). The species of the stage of claim 36 has the media of the species of claims 38-42 (5 species). The species of the stage of claim 43 has the media species of claims 45 and 46 (2 species). The species of the stage of claim 54 has the media of the species of claims 55-60 (6 species). This is a total of 40 distinct species of stages of claim 1 and the media of the dependent claims.

The following claim is generic: claim 1.

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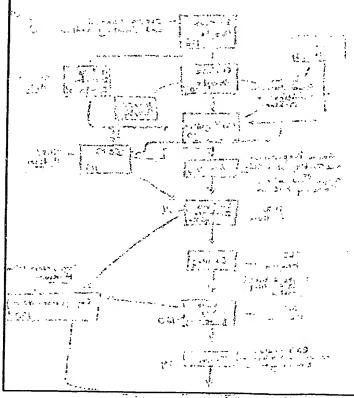
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The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: The stages lack a special technical feature because all of the stages of the IVF process are known in the art. The media used in the method lack a special technical feature because it is well known in the art to modify the media while retaining at least two of the same salts, which fulfills the limitation recited as "a core group" between stages of an IVF procedure. For example, porcine oocyte-cumulus complexes were incubated in NCSU medium containing FF, then incubated in NCSU medium without FF, then the fertilized occytes were incubated in NCSU medium with BSA as taught by Abeydeera et al. NCSU is used as the base medium and would have the same "core salts" throughout the process. The process as claimed lacks a special technical feature, and therefore, lacks unity of invention.



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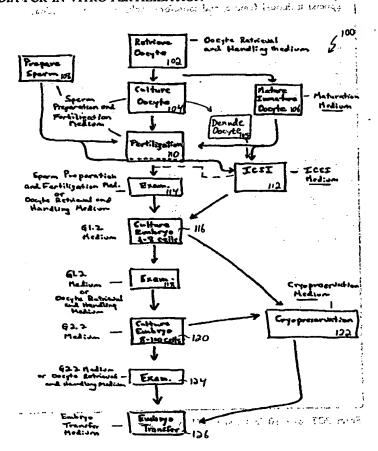
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT, COOPERATION TREATY (PCT)

(51) International Patent Classification 7:	(1	1) Internati nal Publication Number:	WO 00/32140
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 (21) International Application Number: PCT/US2 (22) International Filing Date: 30 November 1999 (3 (30) Priority Data: 09/201,594 30 November 1998 (30.11.9) (71) Applicant: IVF SCIENCES COLORADO, INC. Suite 300, 799 E. Hampden Avenue, Englewood, C (US). (72) Inventors: GARDNER, David, K.; 9927 Clyde Circlands Ranch, CO 80126 (US). LANÉ, Michelle; 16 	99/28408 30.11.99) 8) US [US/US]; CO 80110 :le, 'High- 661 West'	(81) Designated States: AE, AL, AM, AT BR, BY, CA, CH, CN, CU, CZ, I GD, GE, GH, GM, HR, HU, ID, KP, KR, KZ, LC, LK, LR, LS, LT MN, MW, MX, NO, NZ, PL, PT, SK, SL, TJ, TM, TR, TT, UA, UC, ARIPO patent (GH, GM, KE, LS, UG, ZW), Eurasian patent (AM, RU, TJ, TM), European patent (A, ES, Fl, FR, CB, GR, IE, IT, LU, patent (BF, BJ, CF, CG, Cl, CM, NE, SN, TD, TG). Published With international search report. With amended claims and stalement	r, AU, AZ, BA, BB, BG, DE, DK, EE, ES, FI, GB, IL, IN, IS, JP, KE, KG, LU, LV, MD, MG, MK, RO, RU, SD, SE, SG, SI, G, UZ, VN, YU, ZA, ZW, MW, SD, SL, SZ, TZ, AZ, BY, KG, KZ, MD, T, BE, CH, CY, DE, DK, MC, NL, PT, SE), OAPI GA, GN, GW, ML, MR,
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(54) Title: SYSTEM AND SEQUENTIAL CULTURE MEDIA FOR IN VITRO FERTILIZATION

(57) Abstract

Instead of immersing human reproductive cells in a single culture medium throughout the various procedures used in IVF, a process is provided by which the reproductive cells may be moved through a sequence of distinct culture media as the various IVF procedures are carried out. In one implementation, the culture media specifically formulated to provide a physical environment similar to that found within the female reproductive tract and conducive to growth and development of human reproductive cells during the various stages of the IVF process. In this regard, specifically formulated culture media can be applied to support the reproductive cells in one or more of the following procedures: oocyte retrieval and handling; oocyte maturation; ordinary fertilization; oocyte, zygote and embryo examination and biopsy; embryonic development to the eight-cell stage; embryonic development to the blastocyst stage; embryo transfer; and cryopreservation.



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AMENDED CLAIMS

[received by the International Bureau on 19 June 2000 (19.06.00); original claims 1, 36 and 40 amended; new claims 61-127 added; remaining claims unchanged (17 pages)]

A method for use in a human IVF process, wherein the process involves some l. or all of the stages of: occyte retrieval and handling; occyte maturation; sperm preparation; fertilization; oocyte, zygote and embryo examination and biopsy; embryo development; embryo transfer; and cryopreservation said method comprising the steps of:

supporting human reproductive cells in a first support medium during a first stage of said stages, said first support medium including a core group of salts comprising at least two different salts; and

supporting human reproductive cells in a second support medium different than said first support medium during a second stage of said stages, said second support medium including substantially said same core group of salts as said first support medium, said core group of salts utilized in both of said first and second support media thereby minimizing any stress and trauma to human reproductive cells incident to transfer between the first and second support media;

wherein no more than one of said first and second stages is one of said embryo development stage and said embryo transfer stage.

- A method as set forth in Claim 1, wherein said first stage is one of embryo 2. examination and oocyte retrieval and handling.
- A method as set forth in Claim 2, wherein said first support medium 3. comprises water, ionic constituents and a buffer.
- A method as set forth in Claim 2, wherein said first support medium 4. comprises one of 4-Morpholinepropanesulfonic acid (MOPS), N-2-នៃ មេស៊ីវាការិវត្តសិច្ចានា hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES) or bicarbonate! -,7-
 - A method as set forth in Claim 2, wherein said first support medium 4. 10 5. N an vokacioni his 30 comprises carbohydrates. 17
 - A method as set forth in Claim 2, wherein said first support medium 6. xr comprises non-essential amino acids.
 - A method as set forth in Claim 2, wherein said first support medium Standistantial tensor 427.41. comprises glutamine. 345 7 5 St. 250. 4 5 1.50 h77.
- A method as set forth in Claim 2, wherein said first support medium 8. 30 had to . . 30 5 124 18 comprises antibiotics. J. 5 attions and S ac data post of section $\{i,j\}$

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- A method as set forth in Claim 25, wherein said first support medium 34. comprises magnesium and calcium in an aqueous solution.
- A method as set forth in Claim 25, wherein said first stage comprises denuding an oocyte and said first support medium comprises hyaluronidase.
- A method as set forth in Claim 1, wherein said first stage comprises has a process and embryo andevelopment.
 - A method as set forth in Claim 36, wherein said step of supporting reproductive cells in a first support medium comprises supporting a zygote in said first support medium for a time period that is one of at least 48 hours or through at least the eight-cell stage.
 - 38. A method as set forth in Claim 36, wherein said first support medium comprises carbohydrates.
- A method as set forth in Claim 36, wherein said first support medium comprises non-essential amino acids.
 - 15 40. A method as set forth in Claim 36, wherein said first support medium comprises one or more of glucose, lactate and pyruvate.
- A method as set forth in Claim 36, wherein said first support medium To a more than the comprises glutamine. mis 2 60 1 150
- 42. A method as set forth in Claim 41, wherein said glutamine comprises

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 - A method as set forth in Claim 1, further comprising the step of 43. in a third support medium different than said first and second support mediums during a third stage of said stages.
- A method as set forth in Claim 43, wherein both said second stage and 44. 25 said third stage comprise embryo development and transfer.
- A method as set forth in Claim 43, wherein said third support medium is used subsequent to said second support medium and said third support medium has south edicit authors would be the agent of the second of lactate and pyruvate relative to said second o som of an orilomedium of ognor off of enloving the 10-

30 A method as set forth in Claim 43, wherein said third support medium is used subsequent to said second support medium and said third support medium has an elevated concentration of glucose relative to said second support medium.

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81. An aqueous composition, comprising the components:

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ionic constituents sodium, potassium, phosphate, magnesium, bicarbonate, and constituents in water;

a buffer to maintain the pH of said composition within the human physiological range;

the carbohydrates glucose, lactate and pyruvate; and
alanine, asparate, asparagine, glutamate, alanyl-glutamine, glycine, proline, serine and
taurine.

62. The aqueous composition of claim 61, wherein said components are in the form and concentration in millimole per liter, unless otherwise noted, as follows:

NaCl in the range 75-105; KCl in the range 3.5-7.5; NaH₂PO₄.2H₂O in the range of .05-1.5; MgSO₄.7H₂O in the range 0.2-4.0; NaHCO₃ in the range 2.0-10.0; and CaCl₂.2H₂O in the range 0.8-2.8;

15 To the buffer 4-morpholinepropanesulphonic acid (MOPS) with a concentration in the range of 10.0-25.0;

glucose in the range .05-5.0; NaLactate (L-isomer) in the range 5.0-20.0. and

NaPyruvate in the range 0.10-1.0; and

e callent configuration is a clear more aux surgers. In the collection of the paragraph is the range alanine in the range .01-0.5; asparate in the range .01 to 0.5; asparagine in the range .02 to 0.5; asparagine in the range .03 to 0.5; asparagine in the .03

20 0.01-0.5; glutamate in the range .01-0.5; glycine in the range .01-0.5; proline in the range

1973 100 014-0.5; serine in the range .014-0.5 and taurine in the range .01-10.0, and alanyl-glutamine assessment of the property of the second serious and the range of the second serious and the second se

in the range .01-2.0.

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- 63. The composition of claim 62; wherein said buffer is N-2-hydroxyethylpiperazine N'-2-ethane sulphonic acid (HEPES) and has a concentration in the range of 10.0-25.0
- 64. The composition of claim 61, wherein said components do not include calcium or magnesium.
 - 65. The composition of claim 61, further comprising an antibiotic.
 - 66. The composition of claim 61, further comprising at least one human reproductive cell-selected from a group consisting of human gametes, human zygotes and human embryos.
- 10 67. 11 The composition of claim 61, wherein such composition is at least partially contained in a rigid housing.
 - 68. An aqueous composition, comprising the components:

Harmingho and ionic constituents sodium, potassium, phosphate, magnesium, bicarbonate and

range;

a.a. with anothe carbohydratesiglucose, lactate and pyruvate; and silver in

alanine, asparate, asparagine, glutamate, glycine, proline, serine, and taurine.

569 The composition of claim 68, wherein the form and concentration range, in

20 millimole per literaraless otherwise noted, of the components, are as follows:

Provide A tempo though the NaCheer, same and the following the 75-100;

CI 3.5 - 7.5;

NaH₂PO4.2H2O 0.05 - 1.5;

The second second	MgSQ4.7H2Q	. 65 6 0.2 5 4.0 7
	~ 1	
1 4 1 10 1 10 1 1 1 1 1 1 1 1 1 1 1 1 1	NaLactate (L-ison	0.5 - 5.6; mer) 36 16 2:0 20; which are specified at 0.1 - 0.5:
VV 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1)	0.1 - 0.5;
5	NaHCO3	0.1 - 0.5; 15-30\$ CE = 01 Pologram
to a second with the second	CaCl2.2H2O	a to width 0.842.8 }
	Alanine	0.01 #055; 108F 10 to Libitus
	Asparate	0.01 - 0.5;
and the second s	Asparagine	0.01 - 0.5; 0.01-0.5;
10	Glutamate	0.01 - 0.5:
Carting to the second second	GGlycine 1 10 mile	io caliz 0.012.035]
	Proline	0.01 - 0.5:
چار در المواجعة المارية	Serine Serine of	see at 0.01 - 0.5; and a vittocharge :
The state of the s	launne	0.01 - 10.0.
		. e synômes econo.

- 15 70 100 The composition of claim 68, comprising also glutathione.
 - 71. The composition of claim 69, comprising also glutathione in the concentration range, in milligrams per milliliter; of 0.5-5.0; and the concentration with the concentration of the concentration o
- 72. The composition of claim 68, comprising at least one human reproductive cell selected from a group consisting of human gametes, human zygotes and human embryos.
- 20 contained in a rigid housing.
 - 74. The composition of claim 68; comprising also human serum albumin.
 - 75. The composition of claim 68, comprising also hydronate.
- The composition of claim 68, comprising also an antibiotic.
 - 25 An aqueous composition; comprising the compositors:

3.15

ionic constituents sodium, potassium, magnesium and bicarbonate, in water;

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a buffer to maintain the pH of said composition within the human psychological range;
the carbohydrates pyruvate and lactate; and

glycine, proline, and glutamine;

- 5 78. The composition of claim 77, further comprising:
- 10 m 200 2 81.1 The composition of claim 77, comprising also hyaluronate.
 - 82. The composition of claim 77, comprising also polyvinylpyrolidone.
- The composition of claim 77, comprising also hyaluronidase.
- The composition of claim 77, comprising also human serum albumin.
 - 85. The composition of claim 77, wherein the form and concentration range, in
 - 15 millimole per liter unless otherwise noted, of said components are as follows:

NaCi in the concentration range 75.0-105;

KCl in the concentration range 3.5-7.5;

is uno letty of the MgS04. With the concentration range 0.4-4.0;

NaHCO₃ in the concentration range 2.0-10;

20 the buffer MOPS in the concentration range 10-25.0;

CaCl. 2H, C in the concentration range 0.5-2.0;

NaLactate (L-isomer) in the concentration range 5.0-2.0;

5.

NaPyruvate in the concentration range 0.1-1.0; and a service a lanyl-glutamine in the concentration range 0.1-2.0; glycine in the concentration range 0.1-2.0; proline in the concentration range 0.05-2.0; and service a service taurine in the concentration range 0.05-2.0; and service a service taurine in the concentration range 0.05-2.0; and service taurine in the concentration range 0.05-2.0.

- 86. The composition of claim, 85, comprising also hyaluronate in the concentration range 0.02-0.5 milligram/milliliter.
- 87. The composition of claim 85, comprising also hyaluronidase in a concentration range of 0-80 IU/milliliter and human serum albumin in a concentration range 1.0-10 milligrams/milliliter.
 - 88. The composition of claim 77, comprising also at least one human reproductive cell selected from a group consisting of human gametes, human zygotes, and human embryos.
- 15 89. An aqueous composition comprising the components:
 ionic constituents sodium, potassium, phosphate, magnesium, bicarbonate, and calcium, in water;

a buffer to maintain the pH of said composition within the human physiological range;

20 the carbohydrates glucose, lactate, and pyruvate; 1000 12000 ed.

alanine, asparate, asparagine, glutamate, alanyl-glutamine, glycine, proline, scrinc, cysteamine;

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arginine, cystine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, 5.6 . 36 ... threonine, tryptophan, tyrosine, and valine; and 3 0 - 6.5: the vitamins D-Ca Pantothenate, choline chloride, folic acid, i-Inositol, niacinamide, Mills (A. pyridoxal HCl, riboflevin, and thiamine HCl. ·2016年 1895年 1896年 18 14.0 - 7.1) 5 . The composition of claim 89, comprising also human serum albumin (HSA) 90. and hyaluronate. A grants A Real OF B 12.00 - 30.00 Samuel Levil-The composition of claim 89, comprising also at least one component selected 91. 200-12 Antonia likkout from the group consisting essentially of insulin transferin selenium (ITS), insulin-like growth :1-J-10 " T. C. M. 22 ... factor (IGF), and epidermal growth factor (EGF). 1931 C = 113 C o arm tight 92. The composition of claim 89, comprising also follicle stimulating hormone 10 F1 300 - 1000 FR E 1 2 5 (FSH). 10.05 - 0.02: 1 40 1-1 OH 0.0 (c) 1 1.67 36ª The composition of claim 89, comprising also human chorionic 93. bits (Schiller Land) gonadotrophin (hEC). The composition of claim 89, wherein the form and concentration range in 94. แม่ เอหาสาดการรับไป ขา<mark>บก ใ</mark>นเขาสนาไปที่ เพื่อและเปลา " " (ค.ค.ศ. 1 แต่ วัดกรุก (ค.ศ. 17 millimole per liter, unless otherwise noted, of the components are as follows: 15 रहेम्पर मिले बार इक्त द्वाराज १०० है। एक इन्हें के जिल्हा के अपन्य स्टार है। जान 80.0 - 100; bra A.C. - 3.5. 7.5; ್ au 0.05. ≓ 1.5; NaH,PO.2H,O MgSO₄.7H₂O 0.2 - 4.0: 20 NaHCO, 15 - 30.0; m wyses nebasiowaa bas naCaSl₂.2H₂Ohr 12 misloft om it 0.8±2.8 it Glucose 0.5 - 5.5; NaLactates (Leisomer) of 2.0- 20.0; of common a NaPyruvate 0.01 - 1.0;25 Alanine 0.01 - 0.5: ors Asparate 0.01 - 0.5; **CAsparagine** 0.01 - 0.5: Glutamate 0.01 - 0.5;

```
Alanyl Glutamine and Report 0.01102:0; Saluzars
                                                                                                      Glycine
                                                                                                                                                                                                                 0.01 - 0.5;
                                                                                                      Proline
                                                                                                                                             has write the proof of 10:00 and a direction of the
                                                                                                      Serine
                                                                                                                                                                                                                  0.01 - 0.5;
                                                                                                     Cysteamine de la reside and (0.1 - 2.0 junto la reside
                                                                                                      L-Arginine-HCl
                                                                                                                                                                                                                    0.1 - 1.2;
                                                                                                      L-Cystine 2HCl [ ] and a second to 0.05 a 0.25; It is to second
                                                                                                     L-Histidine-HCl-H2O
                                                                                                                                                                                                                    0.1 - 0.4;
                                                                       The Land of Land of the Land of the Color of
         10
                                                                                                     L-Leucine
                                                                                                                                                                                                                    0.1 - 0.8;
                                                                                                     L-Lysine-HCl
                                                                                                                                                                                                                    0.1 - 0.8; તમકાતાલ્યમિક શહે છે છે
                                                                                                     L-Methionine
                                                                                                                                                                                                               0.05 - 0.25;
                                                                       L-Phenylalanine and the 10.1 = 0.4;
                                                                                                     L-Threonine
                                                                                                                                                                                                                    0.1 - 0.8;
15 to take the company of the L-Tryptophane that the finite case 0.1 - 0:9; on query advisor
                                                                                                     L-Tyrosine 2Na
                                                                                                                                                                                                                    0.1 - 0.4;
                                                                                                      L-Valine (40%) said & favo 10.1 -0.8; but (Eff) rotal?
                                                                                                                                                                                                           0.001 - 0.004;
                                                                                                      D-Ca Pantothenate
Choline Chloride And and 0.003 = 0.013
                                                                                                      Folic Acid
                                                                                                                                                                                                         0.001 - 0.0045;
                                                                                                     i-Inositol
                                                                                                                                                                                                             0.005 - 0.02;
                                                                                                     Niacinamide
                                                                                                                                                                                                           0.004 - 0.016;
                                                                                                  Pyridoxal HCl
                                                                                                                                                                                             ____.0.002:≓0.01;∷
                                                                                                      Riboflavin
                                                                                                                                                                                                 0.0001 - 0.0006; and
          25
                                                                                                                                                                                                           0.001 - 0.006) alformations are
                                                                                                      Thiamine HCl
                                                                                     Single Commission of Madeline Commission and the Commission of the
                 The composition of claim 94, wherein the form and concentration range, in
                                               and the constitution in these constitutions are not the constitution of the constitution of the constitutions are constitutions.
                                 milligrams per millimeter, of the components are as follows:
                                                                                              Human Serum Albumin
                                                                                                                                                                                                              1 - 10.9; and
                                                                                         . Hyaluronate
                                                                                                                                                                                   O.H. .. 0.05 40.5.
                                                                                                                                                                                          Martin,7ELC
                                                                                                                                                                                                          Mel City
                                                                                           15-30.3
          30
                                                          96.
                                                                                    The composition of claim 94, wherein the form and concentration range, in
                                                                                                                                                                                                            thus ose
                                 nanograms per milliliter, of the components are as follows:
                                                                                                                                                                                                 PRIVING 4015
                                                                                          ITS
                                                                                                                                                                                                                                                                                                                              1.
                                                                                                                                                                                                     -1 - 100;
                                                                                        GF-I
                                                                                                                                                                                                      10 - 1000; and
                                                                                        FGF
```

Similar (E)

- The composition of claim 94, comprising also the hormone follicle 97. A. 1 - 1 6 stimulating hormone in the concentration range 0.01-10 IU/milliliter. :01 .5.
- The composition of claim 94, comprising also the hormone human chorinonic 98. gonadotrophin in the concentration range 0.01-10 IU/milliliter.
 - An aqueous composition, comprising the components: 99. 23-60

ionic constituents sodium, potassium, phosphate, magnesium, bicarbonate and 3-11-604 calcium, in water; ball (*)

a buffer to maintain the pH of said composition in the human physiological range;

carbohydrates glucose, lactate, and pyruvate; or the house of the ATEE of King and I for the factor

alanine, asparate, asparagine, glutamate, alanyl-glutamine, glycine, proline, senne and 10 of All and the

taurine:

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hyaluronate; and

ทุ่วสำนัญ เอลฟ์ สลาสโดยสัตรสมุเสยกับ ร้าย อ.) การ เกา

COMPORTAL CARGODIAN CARRELATE FOR

angemia en med bere edlig grannet (2)

human serum albumin (HSA).

The composition of claim 99, comprising also ethylenediaminetetraacetic acid 100.

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The Control of the Co

15 (EDTA).

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101. The composition of claim 99, wherein the form and concentration range in the contract of the contract of the grades, are presented in the beautiful to the millimole per liter, except as otherwise noted, of said components are as follows:

.शांक-दर्शकृतकारक कार्य प्रशासित के एक एक प्रशास का कार्य के प्रशास कर के प्रशास कर है। जा है कि स्थापन के प्र

	NaH ₂ PO ₄ .2H ₂ O MgSO ₄ .7H ₂ O	0.05 - 1.5; www.indi	**************************************
çlimandiri, jiyilde, proPek, an	CaCl ₂ .2H ₂ O	0.8 - 2.8;	184 tu :
103.63	Glucose	0.05 -5.0;	4. ***

18:00 %

3 : . .

3

٠,	West State		NaLactate (Lisomer) with NaPyruvate	U.1 - 1.U,
		Plant.	Ole 10,0 agreed and discuss Alanine	் <u>நிற</u> ் நைகையிருக்கின் சி. 0.01 - 0.5;
. 	en mudea	38 %	Asparate Asparagine	7.
		Glutamate Alanyl - Glutamine	0.01 ±0.5; 1 toggad has 4 2	
		1830	Glycine Change and September Proline	0.01 ± 0.5; (**)
10	16 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1		Serine Annual Marine	•
			HSA	1 - 10.0; and
, mari i s	to ay'r ar	$\mathbb{N} = \{_H \}_{t \in \mathcal{T}_{t}}$	Hyaluronate,	0.02-0.5.

102. The composition of claim 99, comprising also EDTA in the concentration range 0.005-0.20 millimole/liter.

of expositions, community port of a recombigation to s

- 103. The composition of claim 99, comprising also at least one human reproductive for a group consisting of human gametes, human zygotes and human embryos.
- 104. The composition of claim 99, wherein said composition is at least partially contained in a rigid housing.
- 20 105. An aqueous composition, comprising the components:

ionic constituents sodium, potassium, phosphate, magnesium, bicarbonate, and the first one is at the first one first one significant assistant and calcium, in water;

a buffer to maintain the pH of the composition in the human physiological range;

carbohydrates glucose, lactate and pyruvate; and

alanine, asparate, asparagine, glutamate, alanyl-glutamine, glycine, proline, and

106. The composition of claim 105, comprising also:

arginine, cystine) histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophän, tyrosine, and valine.

107. The composition of claim 105, comprising also:

D-Ca Pantothenate, choline chloride, folic acid, i-Inositol, niacinamide, pyridoxal

5 HCl, riboflavin, and thiamine HCl.

:7.0 - 1 6

108. The composition of claim 105, comprising also human serum albumin (HSA).

109. The composition of claim 105, comprising also hyaluronate.

millimole per liter, except as otherwise noted, of said components are as follows:

10	MaCI	80.0 - 100;	
	: ***	ジャリグ・みかりるらってら	
	84 10 0 - NaH2PO4.:	2H2O 1 0.05 - 1.5	
	504. ™gSO4.7H	20	
	16104 (NaHCO3	15 - 30 0·	
15	;:0 € - ⊆CaCl2.2H2	0 08-28	
	bas (#966.0 Glucose	0.5 - 5.5:	
	acton - NaLactate (L-isomer) 2.0 - 20.0;	
	NaPyruvate	0.01 - 1.0	
	Alanine	0.01 - 0.5; 0.01 - 0.5;	
. 20 വഴത്തെക്ക്	FR LAZE MEASparate	0.01 - 0:5	Lif
	Asparagine	0.01 - 0.5	
	Glutamate	0.01 - 0.5;	out that it
	Alanyl - Gli	utamine 0.01 2.0	•
n i e many	tellage base of Glycine (3)	audi ? 10 /0.01 - 0.5;	1. 1. 1
25	Proline	0.01 - 0.5: and	1
	Serine : 1111	va iii	ur all y

appeals the tree is only graphers of the also have able to the

111. The composition of claim 105, wherein the forms and concentration ranges on a stronger to need the forms and concentration ranges.

in millimole per liter, except as otherwise noted, of said components are as follows:

		L-Arginine-HCl	e e mieri 4 0.1 #1 ;2 ;#4	<u>ណ្ឌ</u> ន
54.4		L-Cysume Little	0.00 0.20,	
		L-Histidine-HCl _T H	20 - <u> </u>	m. got mair'
		L-Isoleucine	0.1 - 0.8;	
5	•	L-Leucine	17.8.70 more to 10.00 more to	157
			0.1 - 0.8;	
	g Trade - Garage	L-Methionine	∾	50-0
	A. 1944	L-Filchylanamie		
	:	L-Threonine	A. 1 mh 0:1 & 0:8; 1	ento in 100
10	•	L-Tryptophan	0.1 - 0.9;	
	er er er	L-Tyrosine 2Na	30.1 - 0.4; and	.301
		~ 1 cmm.c	(1. X 0. Q.	
	Sign of the	con grad, passe con co	ar to be the training of the	T.69,

112. The composition of claim 105, wherein the form and concentration range in millimole per liter, except as otherwise noted, of said components are as follows:

15	·· •	ι Ν	D-Ca Pantothenate	0.001 - 0.004;
			Choline Chloride	0.003 - 0.01;
			Folic Acid	1.1. (3.3.) ** 0.001 - 0.0045;
		,	i-Inositol	0.005 - 0.02;
			Niacinamide	0.004 - 0.016;
20			Pyridoxal HCl	5 Fig. 5, 0.002 - 0.01;
			Riboflavin	0.0001:- 0.0006; and
		:		2 Nepator 0.001 - 0.006.

- 113. The composition of claim 105, wherein said HSA is in the concentration range of 1-10.0 milligrams/milliliter.
- 25 114. The composition of claim 105 wherein said hyaluronate is in the concentration range of 0.02-0.5 milligrams/milliliter.
 - 115. The composition of claim 105, comprising also at least one human reproductive cell selected from a group consisting of human gametes, human zygotes and human embryos.

- contained in a rigid housing.
- TPA The composition of claim 105, wherein the concentration range of hyaluronate, in milligrams/milliliter, is 0.05-1.0.
 - 5 118 The composition of claim 105, wherein human serum albumin is omitted.
- An aqueous composition for use in human in vitro fertilization, comprising the components:

ionic constituents sodium, potassium, magnesium, phosphate, bicarbonate, and calcium, in water;

- a buffer to maintain the pH of the composition in the human physiological range; and carbohydrates glucose, lactate and pyruvate.
 - 120. The composition of claim 119, comprising also human serum albumin.
- 121. The composition of claim 119, comprising also at least one additive selected from a group consisting of glycerol, ethylene glycol, dimethylsulfoxide, propanedial and surcrose.
- 122. The composition of claim 119, wherein the form and concentration range in millimoles perliter, unless otherwise noted, of said components, are as follows: NaCl in the range 75.0-105; KCl in the range 3.5-7.5; MgSO₄ 7H2O in the range 0.4-4; Na2PO4.2H2O in the range 0.1-1.5; NaHCO3 in the range 2.0-10; MOPS in the range 10.0-25; CaCl2.2H2O in the range 0.5-2.0:

NaLactate (L-isomer) in the range 2-20; NaPyruvate in the range 0.1-1.0; and glucose in the range 0.5-5.5.

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		123.	The composition of claim 120, wherein the cor	ncentration range of human
, e.	•	ι,	serum albumin is 1.0-10 milligrams per millilite	er a sa bezeranên
	1, 17,	124.	The composition of claim 121, wherein the conc	centration range of additives
			glycerol, ethylene glycol, dimethylsulfoxide, ar	nd propanediol is 2 to 20%,
5			and the concentration range for sucrose is 0.1 to	
f 1. <u>t</u>		125.	A system for human in vitro fertilization, comp	prising at least three culture
	media,	includi	ling:	tata-merdian say
	√+3/2€0	(a)	a first culture medium, including:	ja ye yu gerroi
		ionic	constituents sodium, potassium, phosphate, ma	agnesium, bicarbonate, and
10	calciur	n, in wa	vater; no months of a loveled produce live include lan	me) min n
	Ÿ		fer to maintain the pH of said composition with	
· .	range;	Jertha W	and the same of the boles of representations	PO The C
int i i	and the sec	the car	rbohydrates glucose, lactate and pyruvate; and	oeda (SI
÷	m surple	alanin	ne, asparate, asparagine, glutamate, alanyl-glutamin	e, glycine, proline, serine and
15	taurine			esembly of
in the second	1 (4.1744)	(b)	a second culture medium, including:	122 The c
 £.	16 16 Te	ionic	constituents sodium, potassium, phosphate, m	nagnesium bicarbonate and
	19261	n, in w	the graph of the first the first of the firs	vell@
· • 24: .	投标 E()	a buff	fer to maintain the composition at a pH in the hun	man physiological range:
20	95.78 F A	carbol	hydrates glucose, lactate, and pyruvate;	9.00 F
·;	Lineta	alanin	ne, asparate, asparagine, glutamate, alanyl-glutamin	ne, glycine, proline, serine and
-	taurine		ટ. ડે- ડે.ડે જણ <i>દ</i> ાપ ક ોક કરો છક	

land the state of hyaluronate; and so the state of the st

human serum albumin (HSA); and

(c) rea third culture medium, including:

ionic constituents sodium, potassium, phosphate, magnesium, bicarbonate, and

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5 calcium, in water;

maker to maintain the composition at a pH in the human physiological range;

carbohydrates glucose, lactate and pyruvate;

alanine, asparate, asparagine, glutamate, alanyl-glutamine, glycine, proline, and serine;

arginine, cystine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine;

D-Ca Pantothenate, choline chloride, folic acid, i-Inositol, niacinamide, pyridoxal HCl, riboflavin, and thiamine HCl;

human serum albumin; and

hyaluronate.

20

126. The system of claim 125, wherein said first culture medium includes:

ionic constituents sodium, potassium, phosphate, magnesium, bicarbonate, and calcium, in water;

a buffer to maintain the pH of said composition in the human physiological range;

the carbohydrates glucose, lactate, and pyruvate; and

alanine, asparate, asparagine, glutamate, glycine, proline, serine, and taurine.

127. The system of claim 125, wherein said first culture medium includes:

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ionic constituents sodium, potassium, magnesium, phosphate, bicarbonate, and calcium, in water;

a buffer to maintain the pH of the composition in the human physiological range;

the carbohydrates glucosc, lactate, and pyruvate;

5 human serum albumin; and

at least one additive selected from a group consisting essentially of glyccrol, ethylene

glycol, dimethylsulfoxide, propanediol and sucrose the desired medical

and a state of the state of the second secon

रेन्द्र होती के देश प्रसृष्ट कि रोगरेन कुन्यार है. एका अस्ताव है सामग्री के स्थित है से प्राप्त के स्थान

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ता अनेक महिल्ला के अने हो है जिल्ला है। जिल्ला प्रदेश के प्रतिक के विकास

कर्मा विकास के अनुसार के किया के सामान के दूर विकास कर होता कर कराया है। किया के अनुसार कर कराया के अनुसार के

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WO 00/32140 PCT/US99/28408

An amendment to PCT Application PCT/US99/28408 has been submitted simultaneously with this Statement. The amendment replaces claims 1, 36 and 40 with new claims that seek to respond to the observations made in the International Search Report. Claim 1 has been amended to make clear that the embodiment referenced in Claim 1 relates to human in vitro fertilization, as opposed to animal in vitro fertilization, as in Abeydeera, et al., and to indicate that the core group of salts comprises at least two different salts. Additionally, claim 40 has been amended to state that the first support medium comprises three components, glucose, lactate and pyruvate, instead of the two components HSA and hyaluronate.

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T. W. S. W. FELL A

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(54) Title: SYSTEM AND SEQUENTIAL CULTURE MEDIA FOR IN VITRO FERTILIZATION

(57) Abstract: Instead of immersing human reproductive cells in a single culture medium throughout the various procedures used in IVF, a process is provided by which the reproductive cells may be moved through a sequence of distinct culture media as the various IVF procedures are carried out. In one implementation, the culture media specifically formulated to provide a physical environment similar to that found within the female reproductive tract and conducive to growth and development of human reproductive cells during the various stages of the IVF process. In this regard, specifically formulated culture media can be applied to support the reproductive cells in one or more of the following procedures: oocyte retrieval and handling; oocyte maturation; ordinary fertilization; oocyte, zygote and embryo examination and biopsy; embryonic development to the eight-cell stage; embryonic development to the blastocyst stage; embryo transfer; and cryopreservation.



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SYSTEM AND SEQUENTIAL CULTURE MEDIA FOR IN VITRO FERTILIZATION

FIELD OF THE INVENTION

The present invention relates generally to human in vitro fertilization (IVF) and, in particular, to a sequential culture media system and process to be used in oocyte retrieval, handling and maturation, sperm preparation, fertilization, embryo development and transfer, and cryopreservation. The invention provides the gametes, zygote and developing embryo with a physical environment adapted to their physiological needs, so supporting their normal growth and development in vitro and increasing the likelihood of successful pregnancy.

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In vitro fertilization seeks to duplicate, to a large extent, the conditions and processes normally occurring within the female reproductive system that are necessary to pocyte development, fertilization and early embryonic development. In the clinic and laboratory, IVF involves several discrete procedures, such as collection of the oocytes from the ovary of the mother, preparation of the sperm, fertilization, and, once fertilized eggs are identified, a period of early embryonic development, and then transfer of the embryo to the mother's uterus. Each of these steps can take place over extended periods of time; during which the individual cells involved have a continuing need for nutrients, and are subjected to significant stress as a result of clinical manipulation and changed environmental conditions.

During IVF; a culture medium is ordinarily used as a substitute for the fluid secreted by the female reproductive tract that would ordinarily surround the gametes, zygote; and develoring emerge. Most laboratories carrying out IVF use a single culture medium throughout the various procedures involved. In a number of laboratories, there has been a tendency to use tissue culture media for IVF procedures, such as Ham's F-10, which is formulated to support somatic cell growth, not gamete or embryonic cell growth. Tissue culture media generally are complicated systems, containing an array of amino acids, vitamins and other constituents. They can contain components that significantly impair embryonic development and viability after transfer. Further, to the extent tissue culture media contain components that are

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generally needed by the gametes and the embryo, the media are not formulated to provide the components at levels appropriate to support healthy gamete and embryonic development.

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Other laboratories have used simple culture media, consisting of balanced salt solutions supplemented with carbohydrate energy sources such as glucose, pyruvate and lactate. Examples include Earle's, T-6, and human tubal fluid (HTF). These media generally lack certain key components found in the female reproductive tract, such as non-essential aming acids, and their constituents are not formulated in the concentrations that meet the specific changing needs of the gametes and developing the embryo at various stages of their development. Here we have a such as a

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The two types of culture media commonly used for IVF generally are only capable of supporting embryonic development to the eight-cell stage. Such media are ordinarily not capable of supporting and producing a viable blastocyst stage embryo, complete with an epithelium and competent inner cell mass. Accordingly, embryo transfer, the return of the fertilized oocyte to the uterus of the mother, usually occurs at around the four-cell stage (day two) or around the eight-cell stage (day three). This is a time when the four- or eight-cell embryo would not typically have arrived in the uterus of the mother, if fertilization had occurred in vivos. Embryo transfer at this time involves placing the cleavage stage embryo in an environment oriented to a blastocyst stage embryo. The cleavage stage embryo must then undergo further development in a non-homologous environment to reach the blastocyst stage; in which the embryo has trophectoderm cells capable of implanting in the uterine lining.

Recent research and human trials have led to the development of two new culture media, G1 and G2, which represent significant advancements in adaptation of culture media to the physiological needs of the cleavage stage embryo and the embryo in the eight-cell through blastocyst stage of development. These media are described in the following publications: Barnes, Crombie, Gardner, et al. Blastocyst process of the cleavage stage embryo and the embryo in the following publications: Barnes, Crombie, Gardner, et al. Blastocyst process of the cleavage stage embryo and secretical in the following publications: Barnes, Crombie, Gardner, et al. Blastocyst process of the cleavage stage embryo and secretical in the following publications: Barnes, Crombie, Gardner, et al. Blastocyst process of the cleavage stage embryo and the embryo in the eight-cell through blastocyst stage of development. These media are described in the following publications: Barnes, Crombie, Gardner, et al. Blastocyst process of the cleavage stage embryo and the embryo in the embryo and the emb

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Transfer of Human Blastocysts Increases Implantation Rates and Reduces the Need for Multiple Embryo Transfers, Fertility and Sterility, Vol. 69, No. 1, pp. 84-88 (January 1998). Use of these media, and particularly the G2 medium, supports the growth and development of viable blastocyst stage embryos in vitro. Accordingly, the development of these media paves the way for new approaches to embryo transfer to the uterus at the blastocyst stage, when the embryo is most adapted to surviving in the uterine environment and has developed structures and capabilities required for implantation to take place. Research utilizing the G1 and G2 media, and embryo transfer at the blastocyst stage, suggests that these media contribute to higher pregnancy rates, and reduces the need for transfer of multiple embryos and the risk of multiple births. Neither of these media, however, is optimized for supporting the gametes, oocyte maturation, or fertilization.

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specific media and media sequences for supporting gametes, zygotes and developing embryos relative to various phases of the IVF process. In certain respects, such media and sequences better reflect in vivo development. Within the female reproductive system, the oocyte is developed within and released from the ovary during ovulation, and proceeds through the oviduct towards the uterus. During this journey, it experiences a dynamic physical environment. The fluid of the oviduct contains a number of components that provide nourishment to the oocyte and its surrounding cumulus cells, and that also appear to interact with the oocyte and its cumulus cells, so stimulating development. Similarly, the fluid of the female reproductive tract provides nourishment to sperm traveling through the oviduct, and also stimulates certain changes in the sperm traveling through the oviduct, and also stimulates certain changes in the sperm traveling through the oviduct, and also stimulates certain changes in the sperm traveling through the oviduct, and also stimulates certain changes in the sperm traveling through the oviduct, and also stimulates certain changes in the sperm traveling through the oviduct, and also stimulates certain changes in the sperm traveling through the oviduct, and also stimulates certain changes in the sperm traveling through the oviduct and enters the uterus approximately three days later; undergoing internal mansformation and experiencing a changing environment.

As the zygote travels, cell division, or cleavage, occurs as well as significant developmental changes. The cells of early embryonic development have different capabilities and nutritional needs from those of later embryonic development prior to

implantation. The zygote and cleavage stage embryo (up to the eight-cell stage) are characterized by low levels of biosynthesis, low respiratory rates, only limited ability to metabolize glucose, and a capacity to utilize pyruvate. As the embryo develops. and genome activation occurs, the embryo gains an increased capacity to utilize glucose. At the blastocyst stage of development, when the embryo is entering and a within the uterus, the embryo's metabolic system has developed and the embryo has a substantially greater capacity to use and need for glucose; and less need for pyruvate. The makeup of the fluid surrounding the developing embryo in theifemale and angue reproductive tract seems to be tailored to these changing needs; in the oviduct at the time when the oocyte and developing embryo are present, relatively low levels of glucose are found, while pyruvate concentrations are high; at the time the embryo enters the uterus, glucose reaches its highest level and the pyruvate concentration is comparatively low. Cleavage stage embryos, like the oocyte, are susceptible to loss of amino acids through their cell membranes when surrounded by an environment having a low concentration of such factors. Such loss of internal amino acids can have devastating effects. Again, as if in response to these needs of the osmolyte had the sensitive oocyte and cleavage stage embryo, the female reproductive tract typically has high levels of specific amino acids that are very similar to those found in the abuse oocyte and cleavage stage embryo. was the amount of government a good advisor and

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In view of the foregoing, an important object of the present invention is to the further improve and enhance the culture of human reproductive cells in vitro. The invention is intended to promote the health and viability of the garnetes, expote and embryo at various stages of the IVF process, thereby, improving the dverall efficiency: of the IVF process and increasing pregnancy rates.

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In general, the present invention involves the application of separate media or a specifically formulated to meet the physiological needs of the gametes, zygote and/or a developing embryo in various stages of their development, and to support the additional processes necessary to accomplish fertilization and embryonic development invittor. The present invention also generally contemplates a sequential culture media system in which the separate media utilized have integrated formulations, intended to an another during the IVF process.

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In one aspect of the present invention, an oocyte retrieval and handling medium is provided for use in the clinical procedure of retrieving the oocyte from the mother. The medium may be used for flushing, washing and holding the oocyte during the process of removing the oocyte from the mother's ovary, and for storing the oocyte for a period prior to fertilization. An optional use of the medium envisioned by the invention is with procedures where handling or manipulating the oocyte, zygote, or embryo is necessary, such as examination of the oocyte to determine whether fertilization has occurred, or examining the embryo to determine the progression of its development. The present invention includes use of an oocyte retrieval and handling medium comprised of water, ionic constituents, and a buffer. Preferably the buffer used in the medium is 4-Morpholinepropanesulfonic acid (MOPS) or N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES). In addition, the medium may be supplemented with the carbohydrates glucose, lactate and pyruvate. The medium may be supplemented with non-essential amino acids. An optional formulation of the medium, lacking calcium and magnesium, may be used in biospsy procedures. Another optional formulation of the medium includes antibiotics, such as penicillin and/or streptomycin, to destroy bacteria that might be introduced into the medium during the process of oocyte collection.

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Another aspect of the present invention involves the provision and use of an oocyte maturation medium, for example, in circumstances where the oocyte is removed from the mother before it is mature. An example of a situation where application of this medium may be desired arises when it is necessary to treat the oocytes collected from the mother with hormones in vitro due to the mother's intolerance of such normones. The invention contemplates holding the oocytes in the maturation medium for a period following collection of the oocytes, to promote development prior to fertilization. An optional use of the maturation medium in accordance with the invention is for collection, although the most cost effective approach will normally involve use of the retrieval and handling medium for initial flushing, washing, collection and storage, and then transfer to the maturation medium for an extended period prior to fertilization. The present invention contemplates use of a maturation medium comprised of water, ionic constituents, and a buffer. Preferably, the maturation medium is supplemented with the carbohydrates glucose,

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lactate and pyruvate. Specific formulations in accordance with the present invention may involve successive supplementation of the medium with one or more of the following: non-essential amino acids; essential amino acids; cysteamine; human serum albumin (HSA) and hyaluronate; one or more growth factors such as insulin transferin selenium (ITS), insulin-like growth factor (IGF), and epidermal growth factor (EGF); and hormones follicule stimulating hormone (FSH) and human chorionic gonadotrophin (hCG).

Another aspect of the invention involves the provision and use of a sperm preparation and fertilization medium. This medium may be applied to wash, prepare, and store sperm, to store the oocyte in the period prior to fertilization, and to serve as the medium in which the sperm and oocyte are placed together and fertilization occurs. The present invention contemplates use of a sperm preparation and (200) fertilization medium that includes water, ionic constituents, and a buffer. Preferably, the medium contains an elevated concentration of sodium, as compared to the oocyte retrieval and handling medium, to promote sperm function and fertilization. In addition, the medium may be supplemented with an elevated phosphate concentration, as compared to the oocyte retrieval and handling medium. Even more preferably the medium is supplemented with the carbohydrates glucose, lactate and pyruvate, and pyruvate Specific formulations may involve supplementation of the medium with one or more of: bicarbonate; glutathione to promote sperm head decondensation; non-essential amino acids; HSA and hyaluronate; and antibiotics such as penicillin and application was ayem. streptomycin.

A further aspect of the invention relates to a medium for intracytoplasmic sperm injection (ICSI) and related methodology. The ICSI procedure may be necessary where there are obstacles to normal fertilization, such as a thickened zona pellucida on the oocyte hindering sperm head penetration. ICSI involves removal of the cumulus cells and injection of the sperm into the oocyte, ordinarily through a glass pipette. The invention contemplates placing sperm in the ICSI medium, capturing the sperm by drawing the medium containing sperm into the pipette, inserting the pipette containing medium and sperm into the oocyte, and, following insertion into the oocyte, transferring the medium containing sperm from the pipette into the oocyte. The ICSI medium used in the present invention includes the constituents water, ionic

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constituents and a buffer. Preferably, in the present invention the medium lacks phosphate. More preferably, the buffer used in the medium is MOPS or HEPES. Additionally, the medium may be supplemented with the carbohydrates lactate and pyruvate and the medium may be further supplemented with one or more of the non-essential acids most abundant in the oocyte: glutamine, glycine, proline, serine, and taurine. In one formulation, the ICSI medium used is supplemented with hyaluronate or polyvinyipyrolidone (PVP) to slow or immobilize the sperm so that they may be captured by pipette for the ICSI process. Further, an alternative formulation of the ICSI medium referred to as denuding medium used in the invention includes hyaluronidase, which is included in the portion of the medium used to denude the oocyte prior to the ICSI process.

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Another aspect of the present invention involves the provision and use of a medium for supporting initial cell cleavage and embryonic development following fertilization, the medium herein referred to as G1.2. The invention contemplates washing the inseminated oocyte and zygote in the medium and placing the zygote in the medium for a period of about 48 hours to support cell cleavage and development through about the eight-cell stage. The present invention involves use of a medium that includes the constituents water, ionic constituents, and a buffer. Preferably, the medium is supplemented with the carbohydrates glucose, lactate, and pyruvate. The medium may also be supplemented with non-essential acids. Specific formulations in accordance with the invention may include one or more of the following supplements: EDTA, HSA, and hyaluronate. The form of glutamine used in the medium is preferably alanyl-glutamine, which is particularly stable and less prone to breakdown to the waste product ammonium, which is toxic to the developing embryo.

A further aspect of the invention involves the provision and use of a second medium for embryo development, herein referred to as G2.2. The invention contemplates placing the embryo in the G2.2 medium for a period of about 48 hours, preferably at or after the eight-cell stage, and continuing through the blastocyst stage of development and up to the point of embryo transfer. This medium is specifically adapted for and has as its preferred use support of the embryo from the eight-cell stage through the time at which implantation occurs, in tandem with the use of G1.2 for initial embryonic development. The invention involves a G2.2 medium that

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supplemented with the carbohydrates glucose, lactate and pyruvate. More preferably, as compared to medium G1.2, medium G2.2 is supplemented with depressed levels of lactate and pyruvate, and elevated levels of glucose. Additionally, the medium may be supplemented with the non-essential amino acids, except taurine. Specific formulations in accordance with the present invention involve supplementing the medium with one or more of: essential amino acids, which stimulate development of the inner cell mass of the blastocyst; vitamins, which further facilitate the function of the blastocyst; HSA; and hyaluronate. An important aspect of the G2.2 medium, in all formulations, is the absence of EDTA.

Another aspect of the invention is the provision and use of an embryo transfer medium. The invention contemplates that this medium will be used as a carrier for the embryo when it is transferred back into the mother. The invention may involve the same formulations of the medium for embryo transfer as are used with medium G2.2. More preferably for embryo transfer, however, the formulation of G2.2 is supplemented with a higher concentration of hyaluronate, which supports implantation of the embryo in the mother's uterus.

A further aspect of the invention is the provision and use of a medium for cryopreservation of the embryo and/or oocyte. The invention contemplates that the embryo may be placed in the medium at either the one- to eight-cell stage or eight-cell to blastocyst stage, and then frozen and stored in the medium. The invention also contemplates that the medium may be used for cryopreservation of the oocyte. The cryopreservation medium contains ionic constituents, and a buffer. Preferably, it contains the MOPS or HEPES buffer. More preferably, it contains the carbohydrates lactate, pyruvate and glucose. Even more preferably, it contains HSA Most preferably, the medium contains certain additives such as glycerol, ethylene glygol, DMSO, and/or sucrose.

According to a further aspect of the invention, different media are used for two different phases of the IVF process, such as oocyte collection and maturation, sperm preparation, fertilization, embryo development and/or embryo transfer. One associated process involves obtaining a gamete from a first medium and introducing the gamete into a second medium different from the first medium, wherein

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fertilization occurs in the second medium. The step of obtaining a gamete from a first medium may include extracting an oocyte from an oocyte collection medium or oocyte maturation medium as described above. Additionally or alternatively, the step of obtaining may involve extracting sperm from a sperm preparation and fertilization medium as described above which, in turn, may be different from the oocyte medium. The step of introducing the gamete into the second medium may involve introducing the sperm and/or oocyte into a fertilization medium, or injecting sperm into an oocyte contained in the second medium. The various media may have integrated formulations for minimizing trauma to the reproductive cells.

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Another associated process in accordance with the present invention involves obtaining a zygote or embryo from a first medium wherein fertilization has occurred and introducing it into a second medium different from the first medium for a first growth phase. The first medium may be a fertilization medium as described above and the second medium may be the G1.2 medium as described above. The second medium may be used for supporting initial cell cleavage and embryonic development. The method may further involve transferring the resulting embryo from the second medium to a third medium for a second growth phase. The third medium may be a G2.2 medium as described above.

obtaining an embryo from a first medium and introducing the embryo into a second medium different from the first medium for transfer of the embryo into the mother for implantation. The first medium may be a G2.2 medium as described above and the second medium may be an embryo transfer medium as described above.

BRIEF DESCRIPTION OF THE DRAWING

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For a more complete understanding of the present invention and further advantages thereof, reference is now made to the following detailed description taken in conjunction with the drawings, in which:

Figure 1 is a flowchart illustrating an IVF process in accordance with the present invention.

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The following description discloses the composition of various culture media? in accordance with the present invention that are particularly adapted for use with con-IVF. Each of these media is specifically formulated to meet the physiological needs. of the gametes, zygote and developing embryo at key points in the reproductive addition process. Also disclosed is a sequential culture media/system. While each of the shore separate media could be used independently, the media also may be formulated. together as a system, sharing a core group of ionic and non-essential amino acid. constituents, with the objective of minimizing trauma to the oocyte, and the resulting zygote and embryo, as they are moved from one medium to another. The following to description also discloses methods of using the media and the sequential culture media system in various clinical and laboratory procedures by which IVF is carried to out, as well as methods of making the culture media on braces in our in painthous i has gree it almost. The first audience may be ask histories and a green post A. The Composition of the Sequential Culture Media, the artificial results in become made for Doctor Retrieval and Handling Medium of the way your way. A preferred oocyte retrieval and handling medium is an aqueous solution and the second solution and th comprised of the ionic components sodium, potassium, phosphate, magnesium, and description

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comprised of the ionic components sodium, potassium, phosphate, magnesium, medium bicarbonate, and calcium, to maintain an osmotic environment that does not stress the interpretation occyte, and a buffering system, preferably MOPS or HEPES, to maintain the pH of the medium within the physiological range of 7.3 to 7.4. The ionic components are not stress are not stress are not stress are not stress and a solitor and so the medium within the physiological range of 7.3 to 7.4. The ionic components are not stress are not stress and the solitor and solitors are not stress and the solitors are not stress and stress and the solitors are not specifically an applicable as the solitors are not specific and solitors as a stress and the solitors are not specific and solitors as a stress and the solitors are not specific and specific and solitors are not specific and specif

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included in the preferred amounts depicted in column A of Table 1, and may be included in amounts described in the ranges depicted in column B of Table 1.

Table 1

Composition of Oocyte Retrieval and Handling Medium*

	Composition of Oocyte Retrieval and Handling Medium*	
5	Component A Most Preferred	B
		Preferred
	Concentration	Range -
	NaCl Officers State Control of the 90.08	
	KCl. whose specifies months and yes, where the section of the section of	3.5 -7.5
		0.05 - 1.5
	NaH ₂ PO ₄ .2H ₂ O 0.25 MgŠO ₄ .7H ₂ O ²⁴ (2) (4) (4) (4) (4) (4) (4) (4) (4) (4) (4	0.2 - 4.0
10	NaHCO30 Ct at Haw an east president of the distribution of the control of the con	2.0 - 10.0
	MOPS / HEPES 20 CaCl ₂ .2H ₂ O affect to the part that the last to the last	10.0 - 25.0
	2 2	0.8 - 2.8
	settably do detained in the index of an interest management in the second of the secon	0.05 5.0
	Glucose NaLactate (L-isomer) (L-isomer) 10.5	5.0 - 20.0
15	37 B	0.1 - 1.0
		10000. 1
	Alanine (Con a 2016) (ala) Only 1997 House Only 1994 Up with 1998 1995	0.01 - 0.5
	Asparate (asp) 0.1 Asparagine (ash) (ash) (ash) (ash) (ash) (ash)	0.01 - 0.5
20	Glutamated Agriculta (glu) Accordation to the O.1 der they make the Alanyl - Glutamine (ala - glp)	0.01 - 0.5
	Alanyl - Glutamine (ala - gln) 0.5 Glycine (gly) 0.1	0.01 - 2.0
	Proline; 1 of the of i (pro) and an expect of the order of the order.	
	Serine (ser) 0.1	0.01 - 0.5
	Taurine (tau) (tau)	0.01 - 10.0
25	Activities should be specified in the property of the second and the second activities.	· 111
25	* Concentrations are in millimoles unless otherwise indicated; the medium is aqueous.	r . 5 - 7.
	-4-0-0-2.	
	re had not generalished in Thomas at the mother and the presented in this section also. It should be noted that Table 1 and the other tables presented in this section also.	.15.5.5.5.
	It should be noted that Table 1 and the other tables presented in this section also	$ar{ar{\psi}} = ar{ar{\psi}} ar{ar{\psi}} = ar{ar{\psi}} ar{ar{\psi}} = ar{ar{\psi}} ar{ar{\psi}}$
	describe the preferred form of the components used to make the respective culture of the components used to make the respective culture of the components used to make the respective culture of the components used to make the respective culture of the components used to make the respective culture of the components used to make the respective culture of the components used to make the respective culture of the components used to make the respective culture of the components used to make the respective culture of the components used to make the respective culture of the components used to make the respective culture of the components used to make the respective culture of the components used to make the respective culture of the components used to make the respective culture of the components used to make the respective culture of the components used to the compo	5 *
	media in practice. The MOPS buffer has not been used before in IVF procedures	, and
30	is preferred because it is not known to exhibit any toxic effects to reproductive ce	lls
	and does not require maintenance of a CO ₂ atmosphere above the medium. HEPI	
	may also be utilized, although some research indicates a possible toxicity to	
	reproductive cells. Table 1 depicts the preferred amount and ranges for the MOP	S or
	HEPES buffer, although other buffering systems might be used. For example, a	· .
35	hicarbonate buffering system may be used because it is competible with home	$\sigma(0)$

bicarbonate buffering system may be used because it is compatible with human

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reproductive cells. Such a system would not ordinarily be practical with oocyte collection, because it requires the maintenance of elevated levels of CO₂ in the atmosphere surrounding the medium, which is ordinarily accomplished by use of a gassing incubator system that maintains a 3%-10% CO₂ atmosphere. Oocyte collection is a clinical procedure, in which it is typically not possible to maintain an elevated CO₂ atmosphere. In some clinical environments, such as where a humidicrib is available, it may be possible to perform oocyte collection in an elevated CO₂ atmosphere, and a bicarbonate buffer accordingly may be used. In accordance with the present invention, any buffering system used preferably maintains its buffering qualities during exposure of the medium to the atmosphere, and as well is preferably compatible with and not toxic to human reproductive cells.

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The oocyte retrieval and handling medium also includes the carbohydrates glucose, lactate, and pyruvate, at levels similar to those found in the female reproductive tract at the corresponding point of ovulation. The preferred amounts and ranges in which these are found in the medium are depicted in Table 1. In addition, the preferred medium contains Eagle's non-essential amino acids (i.e., those not state) required for the development of somatic cells in culture) alanine, aspartate, asparagine, glutamate, glycine, proline, serine, and taurine, plus glutamine in the form of alanyl-glutamine, at levels similar to those found in the female reproductive system and in the oocyte. The preferred amounts and ranges are depicted in Table 1. The field Same? inclusion of non-essential amino acids and alanyl-glutamine in the medium is SECTION ! important to preventing osmotic shock; a medium lacking these components may drain the oocyte of its internal pool of amino acids, resulting in considerable intracellular trauma. An optional formulation of the medium which may be used in biopsy procedures, omits calcium and magnesium. Another optional formulation of the medium may include one or more antibiotics, such as penicillin and streptomycin, to destroy any bacteria that might be present around the oocyte or that might be introduced through the clinical procedure of oocyte removal. and door but require medicine to be U.O. at the place of the the the contract

2. Occyte Maturation Medium

The oocyte maturation medium is adapted for use with immature oocytes.

Oocyte maturation is typically used with mothers who are unable to withstand the

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hormonal treatment ordinarily employed in IVF. Oocyte maturation generally involves treating the immature oocytes in vitro with the hormones follicle stimulating hormone (FSH) and human chorionic gonadotrophin (hCG) rather than injecting these hormones into the mother. The preferred medium is an aqueous solution that contains ionic constituents similar to those used in the oocyte retrieval and handling medium, 5 at similar concentrations, although the magnesium level is increased and the calcium level decreased to maintain a 2:1 magnesium to calcium concentration. A buffer is included in the preferred medium to maintain a physiological pH. Because oocyte maturation ordinarily occurs in an incubator or isolette in which an elevated CO2 atmosphere can be maintained, a bicarbonate buffering system is preferred. Other buffers may be used, provided they are compatible with the oocyte and other # 1 - 0 G components of the medium. Table 2 provides the most preferred amounts of each of these components, as well as the preferred ranges of these components. 7,1)

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Table 2

	# 21 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	Composition of	Oocyte Maturation Med	lium*
	Comp nent	in which was		el just grinsava et eto trai <u>B</u> Pr ferred
	. (1 · · · · · · · · · · · · · · · · · ·	CONTRACTOR		
		•		ਤਿਹ ਕੁ ਮੁਹਿ ਸੰਬਰੀ ਵਾਲਾ ਸ਼ੁਰੂ <mark>80.0</mark> - 100
_	NaCl			
5	KCl	# N **	0.25	Pertie 2 - 40 - 10 - 10 - 10 - 10 - 10 - 10 - 10
	$NaH_2PO_4.2H_2O$ $MgSO_4.7H_2O$		2	
	NaUCO		25	######################################
	CaCl ₂ .2H ₂ O	ور د کی و د د اش	word ' faribi	11 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
10	~1		4 1 7	07-77
10	NaLactate (L-isomer)	Property of the	Said on 5.87 About h	Sang york, and or a 5 2.0 - 20.0
	NaPyruvate		0.1	0.01 - 1.0
				0.1 - 1.0 2.0 - 10.0 militar emilitar lwee
	Asparate	to the west of	0.1	1997 20 401 (80 H12 0.01 - 0.5)
15	Asparagine	r hitti deelin a	0.1	0.01 - 0.5
	Glutamate		man the O.d. halite	erg /01% of years a (0.01/- 0.5
	Glycine a section	र नेव दक्षार होते ह	्रम्यस्य स्ट्राइ .20.1 राज्यस्य वर्षस्य	(25) 10 (25) 10 (25) 10 (27) 1
20	Serine me Pui	garage Maladata	n kerif 0.1 the or h	
	Cysteamine		0.5	0.1 - 2.0
	L-Arginine-HCl		0.6	0.1 - 1.2
	L-Cystine 2HCl		0.1	0.05 - 0.25
	L-Histidine-HCl-H2O		0.2	0.1 - 0.4 0.1 - 0.8
25	L-Isoleucine		0.4	
	L-Leucine		0.4	0.1 - 0.8 0.1 - 0.8
	L-Lysine-HCl		0.4	0.05 - 0.25
	L-Methionine	•	0.1 0.2	0.03 - 0.23
20	L-Phenylalanine		0.2	0.1 - 0.4
30	L-Threonine		0.5	0.1 - 0.9
	L-Tryptophan		0.2	0.1 - 0.4
	L-Tyrosine 2Na		0.4	0.1 - 0.8
	L-Valine D-Ca Pantothenate		0.002	0.001 - 0.004
35	Choline Chloride		0.002	0.003 - 0.01
33	Folic Acid		0.0023	0.001 - 0.0045
	i-Inositol		0.0111	0.005 - 0.02
	Niacinamide		0.0082	0.004 - 0.016
	Pyridoxal HCl		0.0049	0.002 - 0.01
40	Riboflavin		0.0003	0.0001 - 0.0006
70	Thiamine HCl		0.003	0.001 - 0.006
	HSA		5mg/ml	i - 10.0
	Hyaluronate		0.25mg/ml	0.05 - 0.5
	11, 414101410		.6	
	ITS		10ng/ml	1 - 100
4 5	IGF-I	•	100ng/ml	10 - 1000
-	EGF		100ng/ml	10 - 1000
	FSII	·	0.1U/ml	0.01 - 10
	hCG		C.1U/ml	0.01 - 10
	* Concentrations are in millin	noles, unless otherwise		is aqueous.
	* Concentrations are in millin	noies, uniess otherwise	maicaleu, me medium	is aqueous.

The carbohydrates glucose, lactate and pyruvate are also included in the preferred maturation medium. Because of the presence and importance of cumulus cells that surround the developing oocyte, the glucose, lactate and pyruvate levels are adapted to the needs of the cumulus cells. Non-essential amino acids are preferably included in the medium to provide nutrients and avoid subjecting the oocyte to 5 osmotic stress. Essential amino acids and vitamins may also be included to provide nutrients to the cumulus cells. The medium also contains HSA and hyaluronate, which act as a source of macromolecules. Insulin transferin selenium (ITS), insulinlike growth factor (IGF), and epidermal growth factor (EGF) are included to support 10 the function of cumulus cells, which, in turn, nourish and stimulate the oocyte. FSH and hCG are added to stimulate the cumulus and oocyte to undergo changes associated in vivo with ovulation. It should be noted that, when the maturation and medium is prepared, ITS, IGF, EGF and FSH and hCG are preferably the last-added ingredients. The preferred amounts and ranges of these components are found in 15 Table 2.

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3. Sperm Preparation and Fertilization Medium

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Current methods of in vitro fertilization employ the same medium for sperm preparation and fertilization as is used for embryo development. No attempt has been made to develop a separate medium for preparation of sperm that is also suitable for storage and support of the oocyte, for promoting the process of fertilization, and for supporting the zygotes formed when fertilization occurs. In many laboratories, the fertilization process is allowed to take place over an extended period which ranges from two to three hours to up to about sixteen (16) to eighteen (18) hours. During this time, the oocyte, sperm, and zygotes produced have significant nutritional needs. In addition, sperm function and fertilization tend to be encouraged when the surrounding fluid contains certain constituents. The sperm preparation and fertilization medium of the present invention is formulated to meet these concerns.

A preferred sperm preparation and fertilization medium in accordance with this invention has virtually the same composition of ions and non-essential amino acids as the oocyte retrieval and handling medium. The fact that these media share a similar ionic and amino acid composition minimizes the stress experienced by the

adopted to the readt of the early and the second to the second of herigates

oocyte when it is removed from the retrieval and handling medium and placed in sperm preparation medium. Table 3 sets out the preferred amounts and ranges of the ionic and non-essential acid components.

	· · · · · · · · · · · · · · · · · · ·	A STATE OF THE STA	_
		Table 3 or mg or marke come	Harmon E
5	Composition o	f Sperm Preparation and Fertilization Medi	
	Component	ger i legs sk i 🛕 waa a ladenesti exsel	
	in the state of th	Most Preferred and zeroo which	Preferred
	•	Concentration	Kange
	Show a sufficient of a photo-		
	NaCl (In the American	b focuse (10.6), and $\frac{901}{52}$ and growth forms	78-100
	NC) .	J.J	5.5 - 1.5
		m of a mains cells, w2.06 a money were light	
10	MgSO4.7H2O	e o de de la company de la	0.2 - 4.0
	Glucose	3.15	0.5 - 5.0
		1840 and blanck of 5molectors are over no	
	NaPyruvate	present TLA First 9.32 Red LAT bordering	8t - 17 0.1 - 0.5
	NaHCO3	. 25	13-30
		. ค. 15 (1) รูบการ์สมาศาสนายศาสนาชาติ (1)	
15	CaCl2.2H2O	1.8	0.8 - 2.8
			0.5.50
	Glutathione	1.0mg/ml	0.5 - 5.0
	mos.	on the contract of the contrac	0.012-0.5
	2 22-22-22	one on the Monthly rain shallow won	
20	Asparagine Glutamate) and freshbration as is $rac{1}{2} rac{0}{2} e_{i+1}$	0.01 - 0.5
20	Gluciani	valor a reparate to stiffe or propare e dojan	
	Proline Serine	support of the cocyte 10 promoting the m	0.01 - 0.5
	Serine	் நடிக்கிற முர்கள் மிரிக்கி நூரு நார் தவத்தி அர	0.01 - 0.5
	•		
25	HOAD JOSEP STOP BOTTON BOTTON	रते ता प्रशासका दिन्दा हो at bewolfe से स्टब्स्ट ल्या 'Smg/ml	1.0 - 10.0
23	Hyaluronatë e sel 1932 despit s	sulfile hours to up failing. Of much sold is	0.022 0.5
	Penicillin	0.06mg/ml	001=10
	Streptomycin	0.05mg/ml	0.0110
	and have many services and a first study	era thacton and fertilization lead to be see	
	* Concentrations are in millimole	es unless otherwise indicated: the medium i	Samuel mili
30	to min the constraint to the rest aqueous.	es unless otherwise indicated; the medium i	3E-000 1010
	W	o super a la manda est le ast en	we saged
		ation medium contains sodium at a higher	
	concentration than the level found	d in the cocyte retrieval and handling media	im This

concentration than the level found in the oocyte retrieval and handling medium. This elevated concentration of sodium promotes sperm function and fertilization, without causing undue osmotic stress to the oocyte. There is also a higher concentration of

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phosphate, as compared to the oocyte retrieval and handling medium. The glucose concentration of the sperm preparation and fertilization medium is elevated over that of the oocyte retrieval and handling medium, because glucose is the primary nutrient for sperm and cumulus cells around the egg. The lactate concentration of the present medium is lower than that found in the oocyte retrieval and handling medium, to compensate for the tendency of sperm cells and cumulus cells to give off lactic acid as a waste product. A buffering system is used to maintain the physiological pH, and because sperm preparation and fertilization largely occur within an incubator that can maintain an elevated CO2 atmosphere, a bicarbonate buffer is preferred. Glutathione (not present in the oocyte retrieval and handling medium) is included, to assist in the process of sperm head decondensation. Alanyl-glutamine (present in the oocyte retrieval and handling medium) is omitted from the present medium because it can impair sperin function and reduce fertilization. The same is true of the chelating agent EDTA, which (as will be discussed later) is present in the embryo development media. HSA, the most abundant macromolecule in the Fallopian tube and uterus, is included to support sperm and embryo function. Hyaluronate, which promotes sperm motility, and works in tandem with HSA, is also included. Because sperm tends to contain high levels or bacteria, one or more antibiotic substances are also included. Penicillin, streptomycin, and/or gentamycin are preferred antibiotics. Table 3 sets out the preferred amounts and ranges for these various components.

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In circumstances where it is desired to accomplish fertilization by other than natural interaction of sperm and oocyte, such as where the sperm is unable to fertilize the oocyte due to a thickened zona perfucida surrounding the oocyte, or where the sperm is from a male-factor patient, the sperm may be transported into the oocyte by a technique called intracytoplasmic sperm injection (ICSI). When the ICSI technique is used, the cumulus cells are removed from the oocyte, and sperm is injected into the oocyte's interior using a glass pipette. The present invention contemplates use of a single medium to bathe the oocyte and also to serve as a carrier for the sperm as it is transported by injection into the oocyte. The medium, accordingly, is preferably highly compatible with the interior and exterior of the oocyte. The ionic constituents

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in the preferred medium are similar to those found in the oocyte retrieval and handling medium, except that phosphate is omitted, to avoid metabolic and homeostatic stress. and the magnesium-to-calcium ratio is 2:1. This ratio of magnesium to calcium is felt to be highly beneficial to the oocyte. Because ICSI is a clinical procedure performed outside the incubator, a buffering system that is effective in a normal atmosphere is any used. MOPS and HEPES are accordingly preferred buffers for this medium. Because the cumulus cells have been removed from the oocyte, and the sperm is at the conclusion of its independent life, glucose, the main energy source for cumulus cells and sperm (but not the oocyte) is omitted from the medium. Pyruyate and lactate levels are increased, as these are a primary energy source for the oocyte. Only the non-essential amino acids most abundant in the oocyte - glycine, proline, serine and taurine - and glutamine (in the stable form alanyl-glutamine) are retained in the analysis medium to avoid osmotic and pH stress and to nourish the pocyte. Preferably, the ICSI medium also includes hyaluronate or polyvinylpyrollidone (PVP), to immobilize or slow the sperm so that they may be captured in the ICSI pipette. Table 4 sets out the preferred amounts and the ranges of these components in the ICSI medium. Solveiber Moreover, an alternative formulation of the ICSI medium includes hyaluronidase, without which alternative formulation is used to pretreat the oocyte, to break down the hyaluronate gel holding the cumulus cells around the oocyte. This medium is referred to above as denuding medium, and lacks hyaluronate and PVP but includes in the second hyaluronidase. The composition of the denuding medium includes the constituents of the ICSI medium (except hyaluronate and PVP) in the preferred amounts and ranges shown in Table 4 plus hyaluronidase in a preferred about of 40 JU/ml and a preferred range of Oct-80. Optionally, HSA may be included in the denuding medium in the preferred amount of 5mM, and the preferred range of 1.0 10mM some of 500 000 onto

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	Composition of Medium ICSI*
	Compon nt
	With the second and the Most Preferred Preferred Preferred
	me sort a world seek the engine first it along . Concentration
5	NaChina and red assert below to dispose from the 190.08 and to 75.0 - 105 KCl KCl MgSO ₄ .7H ₂ O 2 2 40.4 - 4 1388
	NaHCO _{3) equicion i trophing resolutivo primer sprimer se 5 to equipe en la Title 2.0 - 10 p. MOPS / HEPES 20 10 - 25.0 combine minute in manuscipino estimation en la tiena de la territoria en la tiena de la territoria en la te}
	CaCls.2H2O we reminded most to find to the sound of the translation of
10	Nalactate (L-isomer) of a little of the following the first section of t
	Government to a service with the grant of the control of the service of the servi
15	Alanyl - Glutamine 0.5 0.1 - 2.0 Glycine ⁶ C to a of confederal brother transfer to 0.5 0.1 - 2.0 Proline Islanda dua cabire and the confederal transfer to 0.1 0.1 - 2.0 Serine 0.1 0.05 - 2.0 Taurine and of corona long it is a series of the corona o
	HSA Hyaluronate of the soft of the state of
	ribered and effected on the experience of a domain that are desired in
20	* Concentrations are in millimoles unless otherwise indicated; the medium is

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aqueous.

5. Embryonic Development Medium G1.2.

The present invention includes an embryonic development medium G1.2. The preferred application of this medium is to support development of the early one-toeight cell embryo. As depicted in Table 5, the preferred medium has a backbone of ionic constituents and non-essential amino acids that is similar to that found in the oocyte retrieval and handling medium. Unlike the oocyte retrieval and handling medium, the G1.2 medium contains the component EDTA, which supports embryonic development and is believed to bind and disable toxins that might have a deleterious effect on the early embryo, and which also suppresses glycolysis. In addition, this

medium includes HSA and hyaluronate, in concentrations that are thought to support early embryonic development.

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The preferred formulation of medium G1.2 differs from the previously published medium G1 in several important respects. First, research has shown that an elevated phosphate concentration may not provide optimal conditions for growth of the the developing embryo. Accordingly, the phosphate concentration has been decreased. Second, hyaluronate has been added to work in tandem with HSA. Third, alanyl-glutamine has been substituted for glutamine. A significant problem for Division embryo culture with amino acids is the natural decomposition of amino acids to ammonium, which decomposition is accelerated at higher temperatures such as the physiological temperature (37 degrees Celsius) used in IVF procedures. Ammonium can be toxic to embryos. Moreover, glutamine is especially prone to decomposition to ammonium within solution. Since embryos are generally cultured in medium G1 or G1.2 for an extended period of up to about 48 hours, a significant quantity of ammonium can develop in the medium and be a significant inhibitor to embryo A. 5 46.5 development. Accordingly, the use of alanyl-glutamine provides substantial advantages; it is a particularly stable form of glutamine and is not prone to breaking down in solution. Also, the concentration of alanyl-glutamine in G1.2 has been ϵESA reduced to .5 mM. These three modifications make G1.2 a significantly improved in the control of medium for early embryonic development over medium G1. The most preferred amounts and preferred ranges of the components of medium G1.2 are depicted in Table 5. Les rain le compositaire le codio esclue enformation ai me acoles maceles de Coloniales.

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	and the second second second	numbers in the second of the s	Table 5	
	Component -	Composi	A.	·
	E crossing ermise	simportur emiliana tere a re	Most Preferred	Preferred
	v 19 a iibae	um lipragram en 'ny jump	C ncentration	Range
5	KCl	សមាលក់ ៩៨ (C) ខ្លះការប្រាក់ សេក	90.08 to 25	80.0 - 100
	NaH.PO. 2H.O	ria, no program wis	3.3 0.35	3.5 - 7.5.
				0.05 - 1.5
	Name ().		2-	
		व्यक्ति । संस्कृतक स्थल है।		
	CaCl ₂ .2H ₂ O	gurges bit go 2 ex	21.8 W. 12.18 C. C. S. C.	0.8 - 2.8
10				0.05 -5.0
10	Nal actate //	icomon)	0.5	0.05 -5.0
	NaPyruvate	women in the second of	97 USS (31 0.5 For process)	5.0 - 20.
	ੀ ਨਿਭਾ ਸੀਵਾਈ ਤਾਰ ਹੈ?	or jeanth of madical d2	-ai) G 2000 G C i 71 %.	0.1 - 1.0
	Alanine		0.1	0.01 - 0.5
	Asparate		0.1	0.01 - 0.5
15	Asparagine		0.1	0.01 - 0.5
	Glutamate	3 CAB	0.1	0.01 - 0.5
	Alanyl - Glutamin Glycine	ne 1995 (1971)	0.5	0.1 - 1.0
	Proline	# 1 Table 1	0.1	0.01 - 0.5
20,	Serine	fore Store A.	0.1 0.1	0.01 - 0.5
W.S. J. Maria	Taurine		0.1	0.01 - 0.5
: V		C. Loan (MAC)		0.01 - 10.0
	EDTA	$\mathcal{X}_{\mathcal{G}}(\mathbb{C}^{+})$	0.01	0.005 - 0.20
		5.5		
	- 20.0 - HSA	₹1.0	<i>5</i> / 1	1.4.100
7.4 0.6	Hyaluronate		5mg/ml 0.1 mg/ml	72H + 721 - 10.0
	3.7		o.i mgmi	(OU) 0.02 - 0.5 USDI ORWO
25 _{C.E}	* Concentrations a	re in millimoles unless otl	nerwise indicated: the	medium issacrati
0.03	aqueous.	187		world in a some
	- 70.C			20 1 1 1 TO 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
		bryonic Development Med		<u>-</u>
	- 10.0 Medium G2	2.2 is also formulated to su	pport embryonic dev	elopment. Its
	preferred use is wit	h embryos from the eight-	cell to the blactooust	100 (and 100
30 ³ S	cells) to around	hundred and	our to the biastocyst	stage (around 100 mattheway 1 - 1 metros of the
		e-hundred cell stage. The		
₹ 4+	essential amino acid	ds preferably found in med	lium G2.2 is essential	lly the same as used
1.4.	with medium G1.2,	except that the concentrat	ion of alanyl-olytomi	ne has been
- 1 - 1	increased This and	upon the mish of multi-		ne nas pecu
t ' :	meiedsen. This red	uces the risk of subjecting	the embryo to osmot	ic stress as it is

moved from medium G1.2 to medium G2.2. Taurine is omitted because its benefits to the embryo appear to be confined to the period prior to compaction. Glucose, lactate and pyruvate are included as carbohydrates, except that the concentration of glucose is increased, while lactate and pyruvate are decreased, as compared to medium G1.2.

- This modification in carbohydrate levels is in response to the increasing ability of the developing embryo to metabolize glucose as an energy source, and reflects also the observed composition of the female reproductive tract. Eagle's essential amino acids are included in medium G2.2 because they are necessary to stimulate the growth of the inner-cell mass of the blastocyst. Vitamins are added as a group because in
- animal studies they tend to facilitate the function of the blastocyst, including fluid accumulation in the cavity of the blastocyst. Importantly, this medium lacks EDTA.

 The preferred amounts and ranges of the components of medium G2.2 are depicted in Table 6.

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Table 6

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15	* <i>j</i>	Compositi	on of Medium G 2.2*	Alank - Ofthing
\ ,	Component	·.·	<u>A</u>	$_{5.430.1}^{\prime\prime}$ B
	() ()	.3)	Most Preferred	Preferred
Sales of the second	15.fc	()	Concentration	Range
				\$866 a 100
t	NaCl	70.0	90.08	80.0 - 100
	KCl		5.5	3.5 - 7.5
	NaH2PO4.2H2O		0.25	0.05 - 1.5
20 (3)	MgSO4.7H2O	Smyle.	1	6.2 - 4.0
	NaHCO3	h 18.1.1.1	25	Statem 115 230.0
	CaCla aHaO		1.8	0.8 - 2.8
	Glucosealar dichery s	thems, a wille ned, wh	o casta 3.45 or differ to	d was aministration of 5.5 P.S.
	NaLactate (L-isome		5.87	2:0 £20.0
25	NaPyruvate	•	0.1	0.01 - 1.0
23	Alanine	C+2 orange	de son On bevellame	0.01 - 0.5
	Asparate		0.1	0.01 - 0.5 0.01 - 0.5
	Asparagine	of marching to the field is	0.1	0.01 - 0.5
			of the control (Notice of the control of the	we start a set of the first of the set of th
30	Alanyl - Glutamine		1	0.01 - 0.5 0.01 - 0.5
	Glycine Signature	1. 100m - 30 Cart	0.1	0.01 - 0.5
	Proline as ad v		en el se 0.41 girlemia	g 2010a outisa %-001 - 0.5
	Serine		0.1	0.01 - 0.5
	I - Arginine-HCl	हा कर्मा है। इसे वह समित	0.6	ove ,5.1 is arosi
35	1 Cycline 2HCl	e see en al estat de la version de la ve	ni, sidr 0.1 , winedca	in the court of 0.05 = 0.25
55	T-CASUMO TOTOR !	1000 2000 2000 2000	,	

			PCT/US99/28408
L-Histidine-HCl-H2O	•	0.2	0.1 - 0.4
L-Isoleucine	2.5	0.4	0.1 - 0.8
L-Leucine			
1. L-Lysine-HCl		0.4	0.1 - 0.8
	•	0.4	0.1 - 0.8
5 & S - L-Methionine	i en	0.1	0.05 - 0.25
L-Phenylalanine	M3 81	0.2	0.1 - 0.4
L-Threonine	18.5	0.4	0.1 - 0.8
C L-Tryptophan	1 J	0.5	⁷ 0.1 - 0.9
L-Tyrosine 2Na	:. ^	0.2	0.1 - 0.9
10 / L-Valine		0.4	0.1 - 0.8 · ·
D-Ca Pantothenate	$\tau_{\mathcal{R}}$	0.002	0.001 - 0.004
Choline Chloride		0.007	0.003 - 0.01
6 k - Folic Acid	Ī	0.0023	9-750 III 0.001 - 0.0045
i-Inositol	1.5	0.0111	0.005 - 0.02
15 V Niacinamide		0.0082	0.004 - 0.016
🐫 🔭 Pyridoxal HCl	1	0.0049	0.002 - 0.01
· · · Riboflavin	કે.દ	0.0003	0.0001 - 0.0006
Thiamine HCl	. 0	0.003	0.001 - 0.006
· · · · · · · · · · · · · · · · · · ·	er 4	5mg/ml	2000 - 0.000 1 - 10.00 - 0.000
20 Hyaluronate	4.4	0.1mg/ml	57 B C 0.02 - 0.5
200-110	1. 14	ong/mi	0.02 - 0.5

PCT/I ICOO MO 400

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WO 00/32140

7. <u>Embryo Transfer Medium</u>

The preferred embryo transfer medium contains the same formulation of

25 constituents as medium G2.2 except that a much higher concentration of hyaluronate concentration of

receptor on the embryo for hyaluronate and that there is also a receptor for the same and that there is also a receptor for the same and that there is also a receptor for the same and that there is also a receptor for the same and that there is also a receptor for the same and that there is also a receptor for the same and that there is also a receptor for the same and that there is also a receptor for the same and that there is also a receptor for the same and that there is also a receptor for the same and that there is also a receptor for the same and that there is also a receptor for the same and that there is also a receptor for the same and that there is also a receptor for the same and that there is also a receptor for the same and that there is also a receptor for the same and the

hyalurcnate on the endometrium of the mother. Hyaluronate is thought to act like a

biological glue that assists the embryo in binding to the endometrium and, which had

constituents of the embryo transfer medium are depicted in Table 7.

Table 7
Composition of Embryo Transfer Medium*

Component Contact Contact Contact	riges y at ≜ r and you want to consult the	; <u>B</u>
Soul of Horistanactics (C. 1961)	Most Preferred Concentration	Preferred Range
NaCl	90.08	80.0 - 100

^{*} Concentrations are in millimoles unless otherwise indicated; the medium is

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Ą	KCl	\$.6	5.5	(1829-0192-enealist 3.5 - 7.5
	NaH2PO4.2H2O	4	0.25	പ്രച്ച ച് രം0.05 - 1.5
* ¿.	MgSO4.7H2O	*.0	1	## 1940 J 0.2 - 4.0
,	NaHCO3	\$4.64	25	. अन्य का क्या 45 - 30.0
5	CaCl2.2H2O	1.0	1.8	racinoiste (10.8 - 2.8 t
	Glucose	5.0	3.15	මය හැකි.බල්සපහට් 0.5 - 5.5
. :	NaLactate (L-isomer)	•	5.87	ananas (2.0 - 20.0
	NaPyruvate		0.1	. weighting (0.01 - 1.0).
	Alanine		0.1	EMS # 100 TY 0.01 - 0.5
10	Asparate	, C.	0.1	3 to 12 0.01 - 0.5 to
40 0	Asparagine	5. 20	0.1	emme (Lornet r 10.01 - 0.5
	Glutamate	7 2 3 2	0.1	42 F (10 5 F 10 10 1 - 0.5
	Alanyl - Glutamine		1	DESS ∪.0.01 - 2.0
	Glycine	(11.1.1)	0.1	.031 ~ 2 0:01 ~ 0.5
15	Proline	\$20000	0.1	eligranic 0:01 - 0.5 (1)
3	Serine	02/00/0	0.1	(CV) takoh 0:04 - 0.5
11	L-Arginine-HCl	₹1 (az v	0.6	mostio 4 - 1.2
29	L-Cystine 2HCl	**************************************	0.1	10/11 to 10:05 - 0.25
3.3	L-Histidine-HCl-H2O	Jan Stephen	0.2	0.1 - 0.4
20	L-Isoleucine	1 g. 1)	0.4	20 10 1 0.1 - 0.8 115
-	L-Leucine		0.4	0.1 - 0.8
	L-Lysine-HCl	San San San San	0.4	0.1 - 0.8
	L-Methionine	.2011 - 1 ²		That is a superior 0.05 - 0.25
	L-Phenylalanine		0.2	0.1 بمر 0.4
25	L-Threonine		0.4	0.1 - 0.8
	L-Tryptophan		0.5	0.1 - 0.9
	L-Tyrosine 2Na			ে 0.1 - 0.4
	L-Valine		0.4	0.1 - 0.8
	D-Ca Pantothenate	Marine Charles (FUSCO)	0.002	0.001 - 0.004
30	Choline Chloride	्रता १० म _् रता विकास	391 1 1 19 00 0 XXX 2	110.0 5.809.0 state as medium G2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2
	T 1' A ' 1		61 613 1 7 4	o namuti ed in. b. 0.001 - 0.0043
	i-Inositol	ASSESSMENT OF THE PARTY OF THE	0.0111	0.003 - 0.02
	Niacinamide Annual Control	ಜನ್ ಕರ್ಮದ	(1. Den. (0.0084) Eve	1391 og dem sid no 0,004 5 0.016 0.002 - 0.01
	Pyridoxal HCl	a a consort to who	0.0049	comobile and see 20002 20.006
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	Thiamine HClaus 1275 and	mobre ari, oi prii	A dela tita A Constitution	5 et 1884 unit en ly 109,001 ± 0.006
	Hyaluronate 1974 Speet	tonin chabon	0.25mg/mi	क्रीलुक्त संतर्भवृत्यः अस्तिसं <mark>0:05</mark> 001.0
	·	ta i <i>ri</i> iyat wab a	SDES OF PROPERTY OF	cosmissees of the entirely an
:	*Concentrations are in m	illimoles, unless	otherwise indicate	ed; the medium is

aqueous.

Cryopreservation Medium at he costa country 8.

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The present invention involves a separate medium to be used in cryopreservation of the oocyte and embryo. The preferred formulation to be used includes ionic constituents and a buffer, preferably MOPS or HEPES, as well as the $\frac{10.5 \text{ M}}{10.5 \text{ M}}$

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carbohydrates lactate, pyruvate and glucose. Optionally, HSA may be included. In addition, the medium may include certain additives, glycerol, ethylene glycol, DMSO, propanedial, and/or sucrose. The preferred amounts and ranges of the constituents of the cryopreservation medium are depicted in Table 86.

	क्टी १४४ होता १ वस्याव सुप्राणि क्रीफार छ।		, , ,
5	twitte beweiten highweiter Afrabi	Table 8	ing the second of the second o
	gradicated the cost to Composition of	of Cryopreservation Medium*	•
	Components Proposition with the components of th	k kagzilens filk i <u>A</u> ntion og 1 ²⁸	<u></u>
	wis or his require soluble per that per cur	Concentration	Preferred Range
	NaCI	90.08	75.0 - 105
	្នាក់ ខ្លាស់ ខ្លាស់ 	on the street of the ine media	
10	the balastic recorder of the arm of balance MgSO ₄ , 7H ₂ O		
	a les vocases del bili a desto granto de la	Lins ruckal inglike, end on E	0.4 - 4
	NazPO4.2H2O · Equal Secondag reduction in a notigated	m of the second of the second	0.1 - 1.5
	tandon in the supportaint modes had	strices are subjected to	
	NaHCO nriw sua Loi tamedar y ann	1 1 1 1 2 5 mg - 1 1	2.0 - 10
	MORS/HERES: ANDVIOLENCE TO THE A		
	alem gradadi ing IVB ma		
	CaCl ₂ .2H ₂ O	•	o from 0.5 ≥ 2.0
		•	
15	NaLactate (L-isomer) Salavia H. 2	4 4 1 5.87 3.00 P	2.0 - 20
	NaPyruvate The best multi est at authoric		
		0.32	0.1 - 1.0
	Glacoca tivi si and ayear similar est.	and all the original value	11V00 (6. 0.4)
	Glucose Springers of the first that of the	and and the property of the control	0.5 - 5.5
	HSA to seek and led by a manazyage was	មានក្រុង មានក្នុងស្ការ៉ាន់ មុខ	0.5 - 5.5
20	HSA OUTS IN FOR ORIGINAL SATE AND REPORT OF STREET	ទោយក្នុង ស្ថាស់ និងក្រៅទាំ សូវ៉ា () 5mg/ml ទីសុស្សនិងសែល និងក្រាស់ និងស្មាស់ ទីភា ពីសេស្សនិងស សមិស្សសិស្ស និងក្រុង និង	0.5 - 5.5 \$\frac{0.5}{1.0} - 10 \$\frac{1}{1.0} - 10 \$
20	HSA CONTROL OF THE PRODUCT OF THE P	Some of the second and	0.5 - 5.5 27 10.7 6 6 6 10 1.0 - 10 7-9 beach the same series Sucrose
20	HSA OUTS IN FOR ORIGINAL SATE AND REPORT OF STREET	5mg/ml So and/or propanedial and/or single for sucrose is 0.1 to 1M terwise indicated; the medium	0.5 - 5.5 1.0 - 10 7-7 beach
20	HSA outside for paidul and a Team of butter and ADDITIVES a continue of the continue of the Glycerol and/or ethylene glycol and/or DM; Range for all except sucrose is 2 to 20%; ran Concentrations are in millimeters unless other viscoses of the viscose of the	5mg/ml Some of the state of th	0.5 - 5.5 1.0 - 10 7-5 beach. sucrose is aqueous
20	HSA ourself for paidul and a Team of here are ADDITIVES of overloade off conclusion of the Glycerol and/or ethylene glycol and/or DM: Range for all except sucrose is 2 to 20%; rat Concentrations are in millimeters unless oth	5mg/ml So and/or propanedial and/or grige for sucrose is 0.1 to 1M terwise indicated; the medium	0.5 - 5.5 1.0 - 10 The bode is a second se

process by which the reproductive cells may be moved through a sequence of distinct culture media as the various IVE procedures are carried out. In one aspect of the invention, the culture media are specifically formulated to provide a physical in sequence environment similar to that found within the female reproductive tract and conducive to growth and development of human reproductive cells during various stages of the IVF process. In a further aspect of the invention, the specifically formulated culture media can be applied to support the reproductive cells in one or more of the following procedures: oocyte retrieval and handling; oocyte maturation; ordinary fertilization; oocyte, zygote and embryo examination and biopsy; embryonic development to the eight-cell stage; embryonic development to the blastocyst stage; embryo transfer; and cryopreservation. Most preferably, the media will be applied sequentially during each of the applicable stages of the IVF process to which the media have been adapted. It should be noted that there is significant variation among clinics and laboratories as to equipment and specific procedures used to accomplish each of the principal steps in the IVF process. The present invention contemplates that the sequential media and process described herein may be utilized and/or readily adapted for use with the wide variety of equipment and procedures employed in IVF practice. What follows is a second more detailed discussion of exemplary applications of the media during IVF and Cattle 21850 c related methodology:

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1. Oocyte Retrieval and Handling; Embryo Handling

Referring to Fig. 1, an initial procedure in the illustrated IVF process
100 is oocyte removal or retrieval (102) from the mother's ovary. This is typically
performed vaginally using a fine needle attached to and guided by a transvaginal
ultrasound probe. The needle is ordinarily connected to fine Teflon tubing and thence
to an aspiration regulator controlled by a vacuum regulator. The aspirate is collected
in test tubes or other appropriate vessels, containing medium. The medium may be
used to preliminarily wash the needle and tubing, and other equipment used in the
procedure. In some clinical settings, the medium may also be used with a specially
adapted needle to flush the follicle and aid in removal of the oocyte. The medium,
equipment used, and aspirate are maintained, so far as possible, at 37 degrees Celsius.

If a bicarbonate buffer system is used in the medium, the procedure ordinarily is

carried out in a gassed humidicrib or isolette which maintains a 3%-10% CO₂ atmosphere. In the absence of such atmospheric controls, the medium must contain a MOPS or HEPES buffering system.

The illustrated process 100 present invention contemplates that the oocyte retrieval and handling medium may be used in each phase of the retrieval process. The process of using the oocyte retrieval and handling medium may involve washing any equipment that may come into contact with the oocyte during removal from the ovary, and that may be used to aspirate, flush and/or wash the oocyte during the removal and collection process. Following removal from the ovary, the oocyte may be washed with medium. Optionally, the oocyte may be stored in the medium for a period.

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In addition, it is contemplated that the medium may be used during other clinical or laboratory procedures where the oocyte is manipulated or handled, and also in procedures where the embryo is manipulated or handled, especially where these occur outside the isolette. Examples would include examination of the oocyte following retrieval from the mother, examination of the oocyte following the fertilization step, and examination of the embryo to determine whether it has developed the eight-cell stage. In each of these examples, the oocyte/embryo would be bathled in the medium as it is withdrawn by pipette from the culture dish or test tube, and would remain immersed in the medium while examined under a microscope or with other equipment. The illustrated implementation of the invention also contemplates that an alternative formulation of this medium, which is calcium and magnesium free, may be used during biopsy procedures.

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in the event the collected oocytes are immature, the illustrated process 100 envisions that a second medium may be used to support and promote development of the oocyte during maturation (106). The oocyte maturation medium would ordinarily be used to treat and mature the oocyte following a collection procedure, in which the oocyte is retrieved from the ovary using oocyte retrieval and handling medium. The retrieval and handling medium and maturation medium have a very similar backbone of ionic constituents and amino acids and glutamine, such that as the oocyte is moved

process 100 includes immersing the oocyte and surrounding cumulus cells in the maturation medium for a period of about 30-48 hours, or until the oocyte is mature. The illustrated process 100 then contemplates removing the oocyte from the maturation medium and immersing it in either sperm preparation and fertilization medium or ICSI medium for purposes of fertilization.

In accordance with the invention, the oocyte maturation medium may be applied to the oocyte retrieval process (102), in place of the oocyte retrieval and handling medium described herein. Additionally, a conventional culture medium, such as Ham's F-10 or medium TCM-199 with or without a HEPES buffer, may be employed for immature oocyte retrieval and handling, before immersion of the oocyte in the maturation medium of the present invention. Once maturation is complete, the oocyte will be immersed in a medium for ordinary IVF fertilization procedure (110), or will be immersed in an ICSI medium in preparation for assisted insemination through an ICSI procedure (112).

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3. Sperm Preparation and Fertilization and Section of the section

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The illustrated process 100 contemplates that the collected oocytes will ordinarily be washed and immersed in, and allowed a period of pre-incubation culture within, a first portion of the sperm preparation and fertilization medium. This period of pre-incubation culture (104) may last up to about six (6) hours. Oocytes permitted a period of pre-incubation culture typically have higher fertilization rates.

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The process 100 also contemplates that the sperm may be separately washed and stored in a second portion of the sperm preparation and fertilization medium to purge it of bacteria and any other contaminants that may be prosent. Sperm preparation (108) may involve dilution of semen with the medium, centrifugation, and resuspension of the concentrated sperm in a new portion of medium. In the "swim up" method of sperm preparation, the medium containing sperm is centrifuged, the medium is drained off, and a new portion of medium is poured over the spundown sperm pellet. The sperm is given a period to "swim up" into the fresh medium. That layer of fresh medium, containing the more motile sperm, is then poured off and centrifuged, and the process is repeated. In another aspect of the invention, the sperm

preparation and fertilization medium may be used in one or more gradient separation procedures, such as the Percoll procedure. The present invention envisions that the sperm preparation and fertilization medium may be used as the medium in any of the sperm preparation procedures that may be used for IVF.

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Once the sperm is prepared (108), the sperm is then examined and counted while in medium, and a desired quantity is added to the portion of medium which contains the oocyte. The sperm and oocyte are permitted to remain together in the medium for a period of up to several hours, and, in some laboratories, for a much longer period, as long as about sixteen (16) to eighteen (18) hours. The invention further contemplates that, following a period of immersion in the medium with sperm, the oocytes will be removed and examined (114) to determine whether fertilization (110) has occurred. When removed for examination, the oocytes will continue to be bathed in the sperm preparation and fertilization medium if the examination can be conducted in an isolette. If not, then, as noted above, the oocyte retrieval and handling medium may be used for handling and examination of the oocytes.

Technique) orienti appropriation to the Ocyte (ICSI) or in

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In the ICSI process (112), sperm may be directly injected into the cytoplasm of the oocyte through a very fine pipette or needle. The process 100 contemplates washing the sperm with a portion of the ICSI medium containing hyaluronate and/or PVP, and then placing the sperm in the medium. The process 100 further involves drawing a microvolume of the medium containing sperm into the pipette and then the injecting the medium and sperm into the interior of the oocyte.

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The illustrated process 100 further contemplates that the oocyte may be bathed in another portion of the ICSI medium during the ICSI process. An alternative formulation of the ICSI medium may be used, supplemented with hyaluronidase, for denuding pretreatment (105) of the occyte prior to the ICSI process. Pretreatment involves immersing the oocyte in the ICSI medium supplemented with hyaluronidase for a period until the oocyte becomes denuded of all or most of its surrounding cumulus cells. Following pretreatment, the occyte is injected with sperm carried in a separate portion of medium, using an ICSI pipette, as provided above.

After the ICSI injection process (112) is complete, it may be necessary to examine (114) the oocyte to evaluate whether fertilization has been effective and the oocyte is intact and healthy. Examination may occur in the ICSI medium bathing the oocyte, or may occur in the oocyte retrieval and handling medium as described above.

5. Embryonic Development to Eight-Cell Stage ber ber an although a same

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Medium G1.2 is applied to the early embryo, following formation of the zygote. After the zygote is identified, it is washed with medium G1.2, and then immersed in G1.2 medium for a culturing period (176) of up to about forty-eight hours. During this time the embryo undergoes development from the one-cell to around the eight-cell stage, and is removed at about the eight-cell stage. Examination (118) of the embryo may occur in the G1.2 medium, or in the obcyte retrieval and handling medium, as described above.

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6. Embryonic Development to Blastocyst Stage and the medical and an analysis

The illustrated process 100 contemplates that medium G2.2 will be used to culture (120) the developing embryo to the blastocyst stage, preferably from about the eight-cell stage to about the one-hundred-cell stage. The process 100 also contemplates that, once the embryo reaches the blastocyst stage, and assuming that the embryo is judged on examination (124) to be viable; it is removed from the G2.2 medium and prepared for transfer into the uterus. In some laboratories, the G2.2 medium may, optionally, be used for embryo transfer as well. Examination (124) of the embryo may occur in the G2.2 medium or in the occyte retrieval and handling medium, as described above.

7.0-31 Embryo Transfer (1805 vill grift of the fillule (201 of 10 notines, before a

The process 100 contemplates that the embryo transfer medium will serve as a carrier for the embryo as it is transferred (126) back into the mother. The embryo will be bathed in the transfer medium, the medium containing the embryo will be drawn into the transfer catheter, the catheter will be inserted into the mother's uterus, guided by an ultrasound probe, and the medium containing the embryo will be injected into the uterus.

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8. <u>Cryopreservation</u>

The cryopreservation medium may be used for storing, freezing, thawing, vitrification, and warming the oocyte, prior to fertilization. The same medium may be used for storing, freezing, thawing, vitrification, and warming the cleavage stage embryo, as well as the embryo in the eight to one hundred cell stage.

While the present invention has been described in relation to one embodiment, it will be appreciated that the invention may be utilized in numerous additional embodiments and procedures. Such additional embodiments and procedures are within the scope of the present invention, as defined by the claims which follow.

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What is claimed is:

1. A method for use in an IVF process, wherein the process involves some or all of the stages of: oocyte retrieval and handling; oocyte maturation; sperm preparation; fertilization; oocyte, żygote and embryo examination and biopsy; embryo development; embryo transfer; and cryopreservation said method comprising the steps of:

supporting reproductive cells in a first support medium during a first stage of said stages, said first support medium including a core group of salts; and

first support medium during a second stage of said stages, said second support medium including substantially said same core group of salts as said first support medium, said core group of salts utilized in both of said first and second support media thereby minimizing any stress and trauma to reproductive cells incident to transfer between the first and second support media;

wherein no more than one of said first and second stages is one of said embryo development stage and said embryo transfer stage.

- 2. A method as set forth in Claim 1, wherein said first stage is one of embryo examination and oocyte retrieval and handling.
- 3. A method as set forth in Claim 2, wherein said first support medium comprises water, ionic constituents and a buffer.
- 4. A method as set forth in Claim 2, wherein said first support medium comprises one of 4-Morpholinepropanesulfonic acid (MOPS), N-2-hydroxyethylpiperazine-N¹-2-ethane sulphonic acid (HEPES) or bicarbonate.
- 5. A method as set forth in Claim 2, wherein said first support medium comprises carbohydrates.
- 6. A method as set forth in Claim 2, wherein said first support medium comprises non-essential amino acids.
- 7. A method as set forth in Claim 2, wherein said first support medium comprises glutamine.
- 8. A method as set forth in Claim 2, wherein said first support medium comprises antibiotics.

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	A method as set forth in Claim 1, wherein said first support medium i
	free from calcium and magnesium and said first support medium is used in biopsy
	procedures.
	10. A method as set forth in Claim 1, wherein said first stage comprises
5	oocyte maturation. The first property and the state of th
	11. A method as set forth in Claim 10, wherein said step of supporting
	reproductive cells in a first support medium comprises supporting an oocyte in said
	first supportimedium for a time period following occyte collection to promote
	development prior to fertilization.
0	12. A method as set forth in Claim 10, wherein said first support medium
	comprises magnesium and calcium disbursed in an aqueous solution.
	13 A rhethod as set forth in Claim 10, wherein said first support medium
	comprises one or more of non-essential amino acids, essential amino acids,
	cysteamine, human serum albumin (HSA), and hyaluronate.
;	14. A method as set forth in Claim 10, wherein said first support medium
	comprises one or more growth factors such as insulin transferin selenium (ITS),
	insulin-like growth factor (IGF), and epidermal growth factor (EGF).
	15. Amethod as set forth in Claim 10, wherein said first support medium
	comprises one or more hormones such as follicle stimulating hormone (FSH) and
	human chorionic genade hopkin (hCG). A second to the secon
	5016. 30 Amethod as set forth in Claim 1, wherein said first stage comprises
	one of sperm preparation and fertilization.
	17. A method as set forth in Claim 16, wherein said first support medium
	comprises carbolity frates bise contributed and the architecture of the contribute of the contributed of the
	18 A mother disposed Could Control of State Control

18. A method as set forth in Claim 16, wherein said first support medium comprises one or more or bicarbonate, glutathione, HSA and hyaluronate.

19. A method as set forth in Claim 15, wherein said first support medium comprises antibiotics.

20. A method as set forth in Claim 16, wherein said first support medium comprises nonessential amino acids.

21: A method as set forth in Claim 16, wherein said first support medium is free of EDTA.

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<i>i :</i>	22.	A method as set f	orth,in Claim	1, wher	ein said firs	t stage comprises
oocy	te retri	eval and handling and	said second	stage co	mprises on	e of sperm
prepa	ration	and fertilization.				PSH-1-CART

- 23. A method as set forth in Claim 22 wherein said second support medium has an elevated concentration of sodium as compared to said first support medium.
- 24. A method as set forth in Claim 22, wherein said second support medium has an elevated concentration of phosphate as compared to said first support medium.
- 25. A method as set forth in Claim 1, wherein said first stage utilizing said first support medium is part of a process of intracytoplasmic sperm injection (ICSI).
- 26. A method as set forth in Claim 25, wherein said ICSI process? comprises removing cumulus cells from an occyte, incubating sperm, and injecting the sperm into said oocyte; and toy and (1964) method is manes as a factor.

said method further comprises the step of placing the sperminjected oocyte into said second grants are to be a local constraint two as such to one stable support medium.

- used in said ICSI process is free from phosphate.
- 28. A method as set forth in Claim 25, wherein said first support medium used in said ICSI process comprises one of MOPS process, HEPES and bicarbonate.
- 29. A method as set forth in Claim 25, wherein said first support medium used in said ICSI process comprises carbohydrates date in the last had an A said in the last had a said in
- 30. A method as set forth in Claim 25, wherein said first support medium as used in said ICSI process is free of glucose. A contract A as bothers A
- 31. A method as set forth in Claim 25, wherein said first support medium used in said ICSI process comprises non-essential amino acids.
- 32. A method as set forth in Claim 25, wherein said first support medium used in said ICSI process comprises glutamine.
- 33. A method as set forth in Claim 26, wherein said first support medium is used for supporting said sperm as part of said ICSI process and comprises one of hyaluronate or polyvinylpyrolidone (PVP).

34: A method as set forth in Claim 25, wherein said first support medium comprises magnesium and calcium in an aqueous solution.

- A method as set forth in Claim 25, wherein said first stage comprises denuding an oocyte and said first support medium comprises hyaluronidase.
- embryonic development.

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- reproductive cells in a first support medium comprises supporting a zygote in said first support medium for a time period that is one of at least 48 hours or through at least the eight-cell stage.
- comprises carbohydrates.
- 39. A method as set forth in Claim 36, wherein said first support medium comprises non-essential amino acids.
- 40. A method as set forth in Claim 36, wherein said first support medium comprises one or more of HSA, and hyaluronate.
 - Amethod as set forth in Claim 36, wherein said first support medium comprises glutamine.
- supporting reproductive cells in a third support medium different than said first and second/supportent diums during a third stage of said stages.
- 44. A method as set forth in Claim 43, wherein both said second stage and said third stage comprise embryo development and transfer.
- 45.2 A method as ser forth in Claim 43, wherein said third support medium is used subsequent to said second support medium and said third support medium has a depressed concentration of one of lactate and pyruvate relative to said second medium.
- 46. A method as set forth in Claim 43, wherein said third support medium is used subsequent to said second support medium and said third support medium has an elevated concentration of glucose relative to said second support medium.

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47. A method as set forth	in Claim 36, wherein said step of supporting
reproductive cells in a first support m	edium comprises supporting an embryo in said
first support medium for a time perio	d that is one of from about 48 to 96 hours and
from about the eight-cell stage to abo	ut the one hundred cell stage. 88 () 188 () 188 ()
48. A method as set forth	in Claim 36, wherein said first support medium
comprises non-essential amino acids	and is free from taurine. memig street pink years
49. A method as set forth	in Claim:36, wherein said first support medium
comprises essential amino acids. 1211	्रात्तरीयको एक दर्गीङ नेप व विष्णु हा उत्यूष्टार वालीका उत्तर मह
50. A method as set forth	in Claim 36, wherein said first support medium
comprises vitamins.	Section 1995 to the second section of the second section of the second s
51. A method as set forth	in Claim 36, wherein said first support medium
comprises HSA.	crum, risen corbobyvirates.
52. A method as set forth	in Claim 36, wherein said first support medium
is free from EDTA.	and their economical curious acidia.
53. A method as set forth	in Claim 36, wherein hyaluronate is added to
	transfer. () A Law , ASA 9 a come to show the state of
54. A method as set forth	in Claim 1, wherein said first stage comprises
cryopreservation.	.emin.string scanq-mar-
55. A method as set forth,	in Claim 54, wherein said first support medium
comprises one of MOPS or HEPES.	Joay types mine.
56. A method as set forth	in Claim 54, wherein said first support medium
	supporting reproductive cells in a third in the com-
57. A method as set forth	n Claim 54, wherein said first support medium
comprises HSA. A large double largery	
grows services	n Claim 54, wherein said first support medium tree
comprises one or more of glycerol, et	hylene glycol, DMSO, proparediol, and sucrose.
59. A method as set forth i	n Claim 36, wherein said first support medium 20 22

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comprises EDTA. The contract of the contract o

A method as set forth in Claim 54, wherein said first support medium hears comprises nonessential amino acids. In the subsequent to make section, supplied to the control of the का महिलान के प्रमुख के अध्यक्ष कर है। कि अपने कि अपने कि कि महिलान कर का क्रियों के क

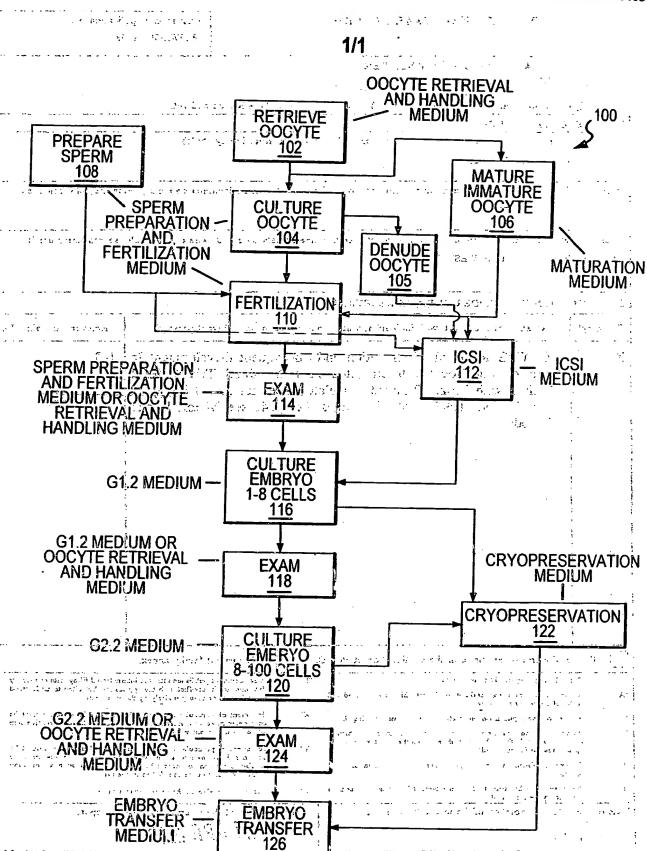


FIG 1

INTERNATIONAL SEARCH REPORT

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International application No. PCT/US99/28408

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A. CLASSIFICATION OF SUBJECT MATTER	· · · · · · · · · · · · · · · · · · ·
IPC(7) :A61F 5/58; A01N 1/02	
US CL :435/2; 600/23, 24, 25 According to International Patent Classification (IPC) or to bot	h national classification and IPC
B. FIELDS SEARCHED	
Minimum documentation searched (classification system follow	ved by classification symbols)
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Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
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C. DOCUMENTS CONSIDERED TO BE RELEVANT.	SSASUMIZE
Category* Citation of document, with indication, where	appropriate; of the relevant passages Relevant to claim No.
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X ABEYDEERA et al. Fertilization an	d Subsequent Development in 1.1-3
Vitro of Pig Oocytes Inseminated	in a Modified I ris-Bullered
Medium with Frozen-Thawed Ejacul	lated Spermatozoa. Biology of
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Further documents are listed in the continuation of Box	C. See patent family annex.
Special categories of cited documents:	"T" later document published after the international filing date or priority
"A" document defining the general state of the art which is not considered	date and not in conflict with the application but cited to understand
to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be
B earlier document published on or after the international filing date	considered novel or cannot be considered to involve an inventive step
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other	when the document is fater along over the claiment of particular reference; the claimed invention cannot be
special reason (as specified)	considered to involve an inventive step when the document is
O document referring to an oral disclosure, use, exhibition or other means	combined with one or more other such documents, such combination being obvious to a person skilled in the art
P document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family
Date of the actual completion of the international search	Date of mailing of the international search report
06 APRIL 2000	18 APR 2000
Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT	SANDRA SANCIED
Washington, D.C. 20231	SANDRA SAUCIER ACTUAL
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT International application No.

PCT/US99/28408

	Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)	
his i	ternational report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:	
· L	Ciaims 1408	
	because they relate to subject matter not required to be searched by this Authority, namely:	
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	because they relate to parts of the international application that do not comply with the prescribed requirements an extend that no meaningful international search can be carried out; specifically:	to anch
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Г	Claims Nos.:	
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	because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.	.4(a). :
i	Observations where unity of invention is lucking (Continuation of item 2 of first sheet)	
s In	emational Searching Authority found multiple inventions in this international application, as follows:	
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International application No. PCT/US99/28408

George Mogu

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING THE PROPERTY OF THE This ISA found multiple inventions as follows:

was at the following the first and the Support to the Control This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:

First, each stage claimed in the method is a distinct species, such as the species of the stage of claim 2, the species of the stage of claim 10, of claim 16, of claim 22, of claim 25, of claim 36, of claim 43, and the stage of claim 54. Further, each medium is a distinct species. For example, the species of the stage of claim 2 has distinct species of media from one of claims 3-9 (7 species). The species of the stage of claim 10 has the media of the species of claims 12-15 (4 species). The species of the stage of claim 16 has the species of the media of claims 17-21 (5 species). The species of the stage of claim 22 has the media of the species of claims 23 and 24 (2 species). The species of the stage of claim 25, has the media of the species of claims 27-35 (9 species). The species of the stage of claim 36 has the media of the species of claims 38-42 (5 species). The species of the stage of claim 43 has the media species of claims 45 and 46 (2 species). The species of the stage of claim 54 has the media of the species of claims 55-60 (6 species). This is a total of 40 distinct species of stages of claim 1 and the media of the dependent claims.

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The following claim is generic: claim 1.

e to a more than the contract of the contract The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because under PCT Rule 13.2. the species lack the same or corresponding special technical features for the following reasons: The stages lack a special technical feature because all of the stages of the IVF process are known in the art. The media used in the method lack a special technical feature because it is well known in the art to modify the media while retaining at least two of the same salts, which fulfills the limitation recited as "a core group" between stages of an IVF procedure, For example, porcine oocyte-cumulus complexes were incubated in NCSU medium containing FF, then incubated in NCSU medium without FF, then the fertilized oocytes were incubated in NCSU medium with BSA as taught by Abeydeera et al. NCSU is used as the base medium and would have the same "core salts" throughout the process. The process as claimed lacks a special technical feature, and therefore, lacks unity of invention.

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